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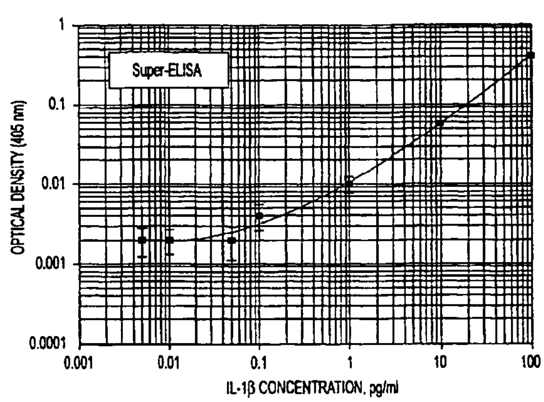
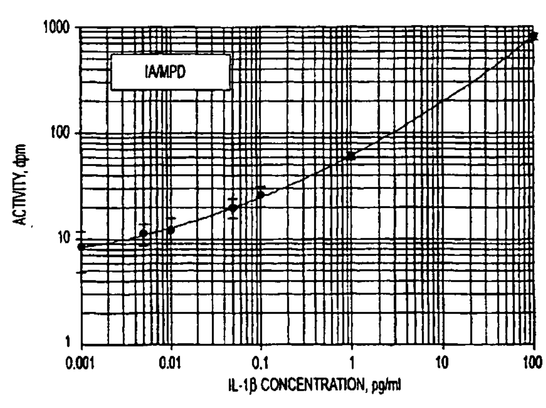
(54) Title: REUSABLE MICROARRAYS FOR QUANTIFYING LOW ABUNDANCE PROTEINS





(57) Abstract: The invention provides protein chip microarrays (P-chips) capable of detecting low abundance proteins from physiologic fluids that exist in concentrations smaller than 0.1 pg/ml. Quantitation is carried out by detecting multiphoton emitting radioisotopes from the assay. The method of multi photon detection provides P-chips with sensitivity of about 50 fg/ml, i.e. about 1,000 fold better than prior art P-chips. Cost effective reusable P-chips and methods of using them are also provided.

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REUSABLE MICROARRAYS FOR QUANTIFYING LOW ABUNDANCE PROTEINS

Field of the Invention

The invention relates to proteomics and the quantitation of proteins in differential abundance in disease states. Specifically the invention provides a microarray for such quantitative assays having features that make it reusable. The invention also provides methods permitting super-sensitive diagnostic P-chips, *i.e.* P-chips in which most targets can be quantitated even if their concentration is less than 500 fg/ml. The superior sensitivity of the P-chips allows them to be low cost, reliable, and reusable.

Background

Genomics and Proteomics are rapidly changing the practice of life science and medicine. Reliable quantitation of rare molecules is necessary to define function at both the cellular and whole organism levels. High throughput is important when dealing with the vast combinations and permutations that determine an individual's genotype and phenotype.

Genomics is the science of deciphering the genetic code by analyzing the mosaic expression of nucleic acid in tissue, sequencing of nucleic acids, analyzing the regulation of nucleic acid expression, mapping of genetic loci, and ultimately determining the encoded proteins of nucleic acids based on comparisons of nucleic acid sequence with others of similar function. Genetic information is stored in the genes of a cell, and this information is mobilized by messenger RNA (mRNA). These mRNAs act as templates for the synthesis of proteins. Modern methods of genomic analysis use DNA microarray technology to measure the presence of specific mRNA molecules to assess gene activity.

Proteomics is both complementary to, and an extension of, functional genomics. Knowledge of mRNA levels, however, does not accurately reflect the presence or activity of the corresponding protein molecules. Proteins are often subject to post-translational modifications and these modifications are often the determinants of activity. The study of proteomics is recognized as necessary for the next decade of biomedical studies and an indispensable extension of Genomics. With the harvesting of biological information resulting from such studies, understanding of biological and pathological processes is attainable. Importantly, the understanding of protein

expression allows practitioners of the appropriate arts to create disease treatments, improve food crop productivity, or bio-manufacture products with commercial importance.

Proteomics can be further categorized into two types: Discovery and Diagnostic Proteomics. Both seek to determine what proteins are involved in a given cellular and/or physiological process and how these proteins change in the course of disease. Discovery Proteomics seeks to understand protein expression at a more fundamental level, that is, the role of post-translational modifications, the interactions of proteins with other bio-molecules, the cellular and organismic signaling events the initiation of cellular processes and how these processes are influenced by stimuli coming from outside of a given cell.

The ultimate goal of Discovery Proteomics is to separate and provide information about the sequences of the previously unknown proteins. The goal is to enumerate almost all proteins in a large number of generic targets: species, tissues, cell lines. The main tools of discovery proteomics are 2D electrophoresis, mass spectroscopy and protein microsequencing by means of the Edman degradation. In contrast, diagnostic proteomics is interested in the elucidation of the presence and/or level of known proteins in a given person, animal or organism. The proteins under study by diagnostic proteomics must be previously characterized by discovery proteomics. A practitioner should have characterized the protein under study and determined the range of abundance of the protein in the wild type population, physical properties of the protein (mass, isoelectric point, functional form), amino acid composition; preferably the sequence, and the dominant post-translational modifications.

Diagnostic proteomics requires high sensitivity analytical methods. Unlike nucleic acids, it is impossible to amplify proteins. The additional complications are post-translational modifications, *e.g.*, phosphorylation, glycosylation *etc*, that occur *in vivo*, but are difficult to reproduce and/or to detect *in vitro*. A fundamental issue in proteomics is, and will be, the sensitivity of the methods to detect and analyze proteins, their modified isoforms and multi-subunit complexes. Immunoassays, Enzyme-linked immunosorbant assay, Fluorescence-activated cell sorting, and other Proteomics technologies known in the art do not fulfill the need of high throughput analysis required by current drug discovery or other commercially important efforts. Since proteins are the important molecules that shape the function and destiny of cells and tissues, and hence are the most prominent targets and products for the pharmaceutical industry, it is necessary to dramatically improve the capability to analyze the "proteome" of cells

and tissues. Therefore successful entrants into this field focus on the emerging field of protein detection and analysis by using a platform of proprietary technologies. We note that practitioners of the art estimate talk of about 100,000 unknown proteins. Thus, it is plausible that the successful proteomics practitioners will specialize in specific sub-fields of proteomics (discovery or diagnostic) stratified either by type of disease or by some sub-group of proteins.

Several years ago, "biochips" were introduced as new tools for informative and cost-effective analysis of both nucleic acids and the proteins encoded thereby. Biochips are specially prepared substrates designed to capture specific nucleic acid or protein molecules in spatially resolved patterns. The captured molecules are labeled and quantitated by an appropriate spatially resolving instrument. Biochips are classified into two subsets: DNA and Protein Chips. RNA can also be used with Biochips as long as some means of stabilization is used in conjunction. Practitioners of the art will appreciate the inherent difficulties of working with RNA, specifically the propensity of single stranded RNA molecules to stabilize by forming secondary structures. As such, the use of more stable nucleic acids are characteristic of preferred embodiments.

In life science and medical practice the use of both DNA and Protein Chips is synergistic. Numerous designs and manufacturing approaches have been implemented for DNA diagnostics but development of biochips for proteins is more problematic. The majority of DNA chips are qualitative only, *i.e.* can not provide the differential display of nucleic acids and typically sensitivity is a few femtomoles/ml. In many biomedical applications the quantitative read-out at attomole/ml level is required.

The DNA and RNA chips are very powerful tools of life science. They permit detection of some abnormalities, especially when the gene encodes a high abundance protein. However, for low abundance, especially molecular switch proteins, the level of RNA often does not correlate with the protein abundance. Thus, when applied diagnostically, the DNA/RNA chips are only partially useful. However, they are much easier to produce and are reasonably user friendly, *i.e.* when diagnostic information is available from DNA/RNA-chips they are favored over more costly P-chips. Thus, in our opinion, the main field of applications of P-chips will be for differentially displayed, low abundance proteins.

Summary of the Invention

Protein chips: The invention provides two types of Protein Chips. Lower sensitivity P-chips permit acquisition of information on hundreds (or thousands) of relatively high abundance, *e.g.*, housekeeping proteins. Low cost is crucial in these operational modes, because the goal is to provide periodic screening, say once a year, to all population of persons above a certain age. Thus the market is large, if one assumes 50 to 100 million tests per year in 2005, providing that the cost is below twenty dollars per test. Thus, we expect an overall market of a few billion dollars per year. We expect that the level of sensitivity of these Screening Protein Chips will be a few pg/ml for any particular target protein. Actually, in some cases quantitation may not be necessary - the most important information may be the presence of a given protein rather than its quantity. The problem addressed by the invention is in low cost production of Protein chips (P-chips).

The inventor estimates that 10 to 20% of Screening Protein chip assays will provide evidence of a disease state in a patient and that, with the help of bioinformatics, such assays may provide even more specific information for the function of the deficient or over abundant protein, thereby suggesting a modus operandi for treatment. Screening Protein Chips should be able to stratify the tested individuals into sub-groups, and suggest the need for advanced checks for microbial contamination, cancer, neuro-degenerative disorders, or autoimmune disease. Thus, the rapid development of Screening Protein Chips (sP-chips) will generate a large market for more sensitive Diagnostic Protein Chips (dP-chips). The inventor estimates that the market for dP-chips is 10-20 million tests per year. Here, sensitivity and reliability are essential and cost is of lesser importance. Diagnostic P-chips may cost $100 per assay or less, and are also useful in therapy monitoring.

In drug validation tests, the leading modem concept is stratification of patients according to their genotype and phenotype characteristics. Recent studies showed conclusively that inclusion criteria for selecting patients for clinical trials according to genetic variances accelerates the drug validation process. It is expected that information about the particular proteome of a given sub-population of patients will further facilitate and improve the drug development and validation process. P-chip based diagnostic tests can be used as inclusion criteria for selecting patients for clinical trials; by predicting safety and efficacy, these tests can enhance the statistical power of a clinical trial. It is estimated that the pharmaceutical industry spends $500-700 million for each

new drug approval and if any significant fraction of the 80% of the compounds that historically fail were approved, even for selected populations, this would have a dramatic effect on fully allocated cost of drug development. This is a model that works in many cases particularly in cancer but also in other disease phenotypes where target specific drug interactions are at issue (*e.g.* neurodegenerative diseases, asthma, and arthritis).

The limitations of previous protein chips: At the recent Conference on Human Proteome Initiative, biologists opined that the geometry and potential number of targets of DNA microarray chips and protein microarray chips will be similar. Specifically they argued that the same well tested "mechanical" devices, *e.g.* bioautomatons sequentially placing specific moieties in a predicted pattern, can be used. The invention shows the limitation of DNA microarray modalities in creating P-chips and provides P-chips created with other better suited methods.

However, the production of reliable P-chips will likely be orders of magnitude more difficult and costly than the production of DNA-chips. The following aspects should be considered when creating P-chips:

- Producing specific moieties to any given DNA are known in the art, while the production of specific antibodies is more difficult;
- DNA probes are more specific and easier to store than antibodies.
- DNA can be reliably amplified whereas proteins cannot;
- the dynamic range of abundance of DNA is a factor of 1-10 whereas the dynamic range of abundance of proteins is close to billion;
- the importance of DNA does not correlate with its abundance, whereas the low abundance proteins are more important diagnostically;
- Many DNA targets (genes) are known but only a few percent of the most abundant proteinsh have been elucidated.

Sensitivity: The current art in P-chips is connected with belief that the high abundance proteins (HAPs) are important diagnostically, providing that one can measure many of them and then use "bioinformatics" to decipher the "significance of changed pattern". Thus, the best previously implemented P-chips try to measure a large number, in some projects a few hundreds with a "futuristic" design for a few thousand proteins. However, the sensitivity of previous P-chips is

inadequate - typically 100-500 pg/ml for majority of low abundance targets. Also, current P-chips are unable to differentiate between different post-translational modifications.

The invention realizes the importance of Low Abundance Proteins (LAPs) versus the High Abundance Proteins (HAPs). First, the level of abundance of HAPs rarely correlate with the disease, *i.e.* HAPS are usually not differentially displayed. Recent results in the field of proteomics suggest that the levels of HAPs correlate well with the level of DNA as measured by DNA chips and the levels of RNA as in transcriptomics. However, the cost of nucleic acid chips is orders of magnitude lower and sensitivity much higher than for protein chips. Thus, for HAPs, the nucleic acids provide much more information at lower cost than P-chips. *Ergo*, for HAPs there is no biologic or economic justification for using P-chips.

The situation differs in the case of LAPs. These proteins are:

- the molecular switches and often play the role of signaling or immunomodulating entities;
- their levels rarely correlate with level of encoding DNA or RNA;
- their levels are differentially displayed and drastically changes in the case of disease;
- consist a class reasonably homogeneous in abundance; their level per cell is typically 100-10,000 copies, *i.e.* only about two logs;

The change of level between one clinical paradigm and another can be very large; using supersensitive MPD enhanced immunoassays the inventor documents that the abundance of an important cytokine, IL-1β is suppressed by a factor of about ten in the case of patients with Acute Myelogonous Leukemia (AML), a known cancer of blood. Also, the recent results suggest that the down-regulation of LAPs may be much more important physiologically then their overproduction.

All these finding are against the current paradigm. The above cited results have been obtained using super-sensitive MPD enhanced immunoassay (IA/MPD), which achieved recently the landmark sensitivity of 1 fg/ml, *i.e.* about 1,000 fold higher sensitivity than prior art immunoassays, including the best ELISAs.

IA/MPD ranged from 1 fg/ml to 100 pg/ml. This is a factor of about 500 increase in sensitivity over a standard ELISA. We studied the influence of different components of nonspecific background on the performance of MPD enhanced immunoassays. The background is dependent on the assay conditions, especially the washing conditions. Also, optimization of pH and use of appropriate blocking procedures is preferred. Only about 30% of background is true non-specific biological background (NSBB) which appears when serum is added. We further minimized NSBB by changing the temperature and the pH at which the biotinylated antibodies are bound. Even though NSBB is important in the serum samples, it only marginally influences the reproducibility of the assay.

Also, for IL-4, IL-6, IL-10 we achieved the LOD = 1 fg/ml with the CV of between 20-30%. Similar results have been achieved for p24 of HIV-1. Comparison of sensitivity of IA/MPD for different interleukins is provided in Table 1.

Table 1: ELISA and IA/MPD Sensitivities For Select Cytokines

Interleukins	Measured serum values (pg/ml)	Reported ELISA LOD(pg/ml)	IA/MPD LOD(pg/ml)
IL-1â	0.007 - 5.5	Other 0.5-3.0 BioTraces 0.05	0.001
IL-4	<1	Other 0.2 -3 BioTraces 0.05	0.001
IL-6	0.01- 20	Other 0.2-1 BioTraces 0.11	0.005
IL-10	0.008 - 10.5	Other 0.5 -5.0 BioTraces 0.075	0.001
IL-11	<2	Other 4-5	0.01
IL-12 (76kD)	<2	Other 0.5-3	0.08

LOD (Limit of Detection) = 0 pg/ml mean value + 2 SD

Other = R&D Systems, Minneapolis, MN; Endogen, Woburn, MA; Genzyme, Cambridge, MA; CLB, Amsterdam, NL

Application of IA/MPD for quantitation of cytokines in clinical specimens: Some biomarkers (cytokines, growth factors, caspases) levels in serum are upregulated during certain disease states, such as IL-6 (inflammatory responses). ELISA provide adequate sensitivity for

measuring levels of biomarkers that are expressed at high, about 1 pg/ml, but lacks the sensitivity to accurately measure levels of biomarkers usually found at trace levels in healthy individuals, *e.g.* IL-1β and IL-10, and down regulated due to the onset of diseases. We believe information regarding down regulation of such biomolecules can improve our understanding of complex physiological process and facilitate the development of therapeutics that modulate these biomolecules for the control of human diseases. Super sensitive IA/MPD can illuminate the potential applications for diagnostic, prognostics and drug development.

In our study we compared side by side ELISA and IA/MPD for quantitation of IL-1â, IL-6 and IL-10. We used a sensitive ELISA developed by scientists at Biotraces for our comparison studies. The sensitivity of IA/MPD for IL-1â, IL-6 and IL-10 has been around 10 fg/ml. Thus we have developed IA/MPD assays with sub-attomole/ml sensitivities for IL-4, IL-11 and IL-12. The sensitivity of those assays ranges from 0.06 to 0.002 pg/ml that is 100 to 1,000 fold improvements over the classical ELISA for these targets. Using IA/MPD levels of IL-1β, IL-6 and IL-10 were quantified in 100 clinical samples. Samples included 40 serum samples collected from AML patients and 60 samples from controls. Each sample was measured in triplicate. We measured the level of IL-1α for which techniques are clearly not sensitive enough.. The results for IL-1α are striking; 70% of control samples show clearly measurable, though very low (< 0.2 pg/ml) level of IL-1â. However, level of IL-1â was down regulated in AML patients and was ***not measurable*** in AML patient even with IA/MPD, which is capable of quantitation at 0.005pg/ml sensitivity. This data strongly suggest that IL-1â may become an important cancer marker. Our results also demonstrated up regulation of IL-6 in AML and confirmed previously reported results. Even though some overlap between healthy and AML patient was observed, we could conclude that low level of IL-6 indicate that an individual does not have AML. Thus, the use of IA/MPD for measurement of IL-6 may present an efficient therapy-monitoring tool for some patients. The data shows that IL-10 is slightly overabundant in AML patients. Also, our data suggest that it will be clinically important to further improve the IA/MPD by a factor of about five.

We discovered an apparent contradiction in AML patients; the levels of IL-1â in blast cells are increased, but the serum levels are decreased compared to controls. As the levels of IL-1â in blasts are a factor 5-10 higher and in serum is about 10 fold lower, we observe the factor fifty modulation of secretion. Only large changes in level of post-translational modification can

explain this result. These results strongly suggest that the activity of Caspase-1, which cleaves pro-IL-1α and pro-IL-18 into their mature forms reduced. Thus, immature IL-1α is not secreted, but is retained within the cells. A major effort is needed to determine the biochemical mechanism by which the activity of caspase-1 is regulated in AML. These will permit a better understanding of the influence of cancers on the network of caspases and apoptosis.

One important conclusion from these worldwide first study of level of cytokines at sensitivity better than 50 fg/ml is that one needs to measure both down-regulation (IL-1â, IL-10) and up-regulation (IL-6). Note, that currently more than 100 cytokines had been discovered and for about 50 cytokines the important physiologic function(s) has been elucidated. These data strongly suggest the clinical importance of P-chips targeting majority (preferably all) cytokines using the dP-chip with a typical sensitivity of better than 50 pg/ml. Note, that the best current P-chip for cytokines under development by Beckman-Coulter has the sensitivity of about 10 pg/ml, *i.e.* about 200 times to low to be clinically important. These orders of magnitude discrepancy between prior art and what is needed clinically calls for bold, innovative redesign of P-chips. This redesign is disclosed in this invention.

Brief Description of the Drawings

Figure 1 provides the comparison of ELISA and IA/MPD for IL-1α. The limit of detection (LOD) of IA/MPD is 0.001 fg/ml, *i.e.* 1,000-fold better than commercially available ELISA assays. The IA/MPD for IL-1α is sensitive and reproducible.

Figure 2 shows the CV for this assay; all points are in sixplet. Note the 40% and 30% CV at 1 and 10 fg/ml, respectively. This is further confirmed by other analysis of the data. The distributions of measurements at level of 10, 100 and 1,000 fg/ml are plotted, and all distribution curves are close to Gaussian with good CV. In each case, the 32 samples have been measured concurrently.

Detailed Description of the Invention

Definitions

Detailed Description of the Invention

Definitions

1. The term "target protein" as used herein refers to a peptide, polypeptide, or protein, which is either monomeric or multimeric. The source of said target protein can be a tissue cell lysate, blood and plasma, excretory fluid, or any physiological fluid originating in an organism wherein and is extracted, isolated, and substantially purified using methods known in the art.

2. The term high abundance protein (HAP) as used herein means a protein that is present at more than about 100,000 copies in a typical cell, which translates into an abundance of more than about 100 pg/ml in blood. For a HAP with a molecular weight of 20,000 Dalton it translates into 5 femtomole/ml.

3. The term medium abundance protein (MAP) as used herein means a protein that is present at more than about 10,000 copies in a typical cell, which translates into an abundance of more 10 picogram/ml (pg/ml) in blood. For a MAP with a molecular weight of 20,000 Dalton it translates into 500 attomole/ml.

4. The term low abundance protein (LAP) as used herein means a protein that is present at about 100 to 10,000 copies in a typical cell, which translates into an abundance of between 0.1 and 10 pg/ml in blood. For a LAP with molecular weight of 20,000 Dalton it translates into 5 to 500 attomole/ml.

5. The term very low abundance protein (VLAP) as used herein means a protein that is present at about 1 to 100 copies in a typical cell, which translates into an abundance of between 1 and 100 femtogram/ml (fg/ml) in blood. For a VLAP with molecular weight of 20,000 Dalton it translates into 5 to 500 attomole/ml.

6. The term "epitope" includes any antigenic determinant or antigenic site that interacts with an antibody, *e.g.*, the Class I- binding peptide compositions used in the methods of the invention. An "antigen" is a molecule that induces the production of an immune response. An antibody binds to a specific conformational domain of the antigen called the "antigenic determinant" or "epitope". The antigens primarily used herein are the target proteins described above. In this

patent, we use the term "epitope" in generalized sense, *i.e.* including any part of protein to which a specific moities bind with good K^d, *e.g.* Abs, "camel" antibodies, specific moieties generated by phage display, specific moieties generated via recombinant methods, specific antibodies generated by recombinant methods and containing the RNA fragment or aptamers Antibodies generated by polypeptides

7. The term "antibody" as used herein includes immunoglobulins of mammalian origin, antigen specific immunoglobulins, polyclonal antibodies, monoclonal antibodies, fusion proteins comprising antigen specific variable region (V region) and viral coat proteins as in phage display libraries, any other protein which contains an antigen specific V region. Phage display libraries are generated by cloning heavy and light chain variable region encoding genes and fusing said clones with viral coat protein encoding genes. Phage expressing specific antibody domains are recovered through selection and used to transfect bacterial hosts to produce high titer lysates of the anitgen specific phage.

The preferred method of producing monoclonal antibodies, known in the art, involves the inoculation of rodent hosts with an antigen of interest and administering booster inoculations about three days after. Spleen cells are harvested and cultured along with an immortalized hypoxanthine deficient myeloma cell line in the presence of polyethylene glycol to facilitate hybridization and formation of hybridomas. Hybridomas are selected by culture in hypoxanthine-aminopterin-thymidime medium.

Hybridoma are screened for antibody production of desired specificity and cloned to yield a uniform cell line which continuously produces said monoclonal antibodies. (*See* Sambrook, J., Fritsch, E.F., Maniatis T.: *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989, *See also* Harlow, E. and Lane, D.: *Antibodies: a Laboratory Manual.* Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1988).

8. The term "aptamer" as used herein includes all synthetic oligonucleotides (i.e. ssDNA, dsDNA, ssRNA, dsRNA, rRNA, or PNA) that can bind a specific molecule (i.e. a protein, peptide, polypeptide, or metabolite). A variety of commercially available oligonucleotide synthesizers can be utilized in aptamer synthesis (*i.e. Perkin Elmer/Applied Biosystems*, Inc. Model 380A, 390B, 394/5, or 394/8 DNA synthesizer, and ABI 3900 High Throughput DNA

Synthesizer, *PolyGen* DNA-10 Column-Synthesizer). We note, however, that the main challenge in development of aptamers is not a production of a library of nucleic acids but selection of the nucleic acids that bind specifically to a given protein. The most efficient way of generating aptamers is *via* the combination of the PCR step with stringent binding selection as described by L. Gold *et al.*, in a series of patents.

9. The term "end point immunoassays" (IA) as used in *current* means an assay which quantitates the abundance of a particular protein in a biologic sample. Typically used format of biomedical diagnostics measures a given protein in 96 samples, concurrently, using the 96 well microtiter plates. Recently, IA in 384 well microtiter plates has become popular.

10. The term "panel of immunoassays" (PIA) as used in *current* means a procedure which quantitates the abundance of a small set of particular proteins in a biologic sample. Typically used format of biomedical diagnostics permitting implementation of "panel of IAs" measures 8 different proteins for 12 different samples. For technical reasons, the number of different proteins measured by panels of Immunoassays is limited to a few, say less than 10 (see discussion above).

11. The term of protein microarray (P-chips) as used in prior art, means a procedure in which a large set of proteins from a particular biological sample is measured in parallel. The smallest P-chips feature 16 different proteins but typically, P-chips attempt to measure in parallel about 100 targets. The largest P-chips in consideration (not yet implemented) will feature a few thousands proteins. The term "protein chip" as used herein refers to a microarray made up of a supporting means to which a probe molecule has been anchored. These probe molecules can be antibodies, aptamers, or a combination of both and are used to screen for some target protein to which the antibodies/aptamers bind with high specificity. Currently, these chips are manufactured *via* automatic "bio-matons" featuring an array of pins which gently place droplets of appropriate buffer containing antibodies onto surface of the solid state material protein chip in a preordered fashion such that a practitioner can track the position of said antibodies.

12. The term "reusable protein chip" as used herein refers to a microarray made up of a supporting means to which a probe molecule has been anchored. The present invention confers an aspect of reusability by the manufacture of microarrays with an excess of probe molecule to target proteins (in a ratio of 1000:1 or greater). Furthermore, the use of short lived radioisotopes

as a mode of labeling allows for subsequent use of the same microarray after previously used labels dim or are extinguished through radioactive decay.

13. The term "reusable randomly addressed protein chip" as used herein refers to a microarray made up of a library of supporting means, *e.g.* beads to which a probe molecule has been anchored. These probe molecules can be antibodies, aptamers, or a combination of both and are used to screen for some target protein to which the antibodies/aptamers bind with high specificity. The said library of solid state objects, *e.g.* beads is coded, *e.g.* optically labeled and a given code is uniquely assigned to one and only one bead. The preferred implementation includes the random distribution of these beads on the surface of appropriate holder, *e.g.* random ordering in an x-y array, either fully occupied or with a fraction spots empty. The read-out generally consists of two operations: establishing the amount of biomolecule on each of bead, and then decoding each bead label. Generally, the first step will use the highest possible sensitivity means, *e.g.* MPD instrumentation described in the following, whereas the step of "reading the code" is a low sensitivity operation and can be accomplished by optical means.

The invention confers an aspect of reusability by the manufacture of microarrays with an excess of probe molecule to target proteins (in a ratio of 1000:1 or greater). Furthermore, the use of short lived radioisotopes as a mode of labeling allows for subsequent use of the same microarray after previously used labels decrease or disappear through radioactive decay.

14. The term "supporting means" refers to a solid phase having a flat or planar, rigid or semi-rigid surface. Preferred means include low cost plastic surfaces. However, to achieve high sensitivity, the said surfaces have to be appropriately derivatized to decrease the non-specific biological background. Other materials for use as supports for P-chips comprise planar crystalline substrates such as silica based substrates (*e.g.* glass, quartz, or the like), or crystalline substrates used in, *e.g.*, the semiconductor and microprocessor industries, such as silicon, gallium arsenide and the like. These substrates are generally temperature resistant, pH resistant, and ion resistant, and generally remain stable when exposed to reagents used in biological and chemical assays. Silica aerogels and other 3D microporous media may also be used but are typically leading to high NSBB. Such aerogel substrates may generally be prepared by methods known in the art, *e.g.*, the base catalyzed polymerization of $(MeO)_4Si$ or $(EtO)_4Si$ in ethanol/water solution at room temperature.

15. The term "MPD" as used refers to multiphoton detection technology used for enhancing detection sensitivity for some radioisotopes. A detailed explanation of MPD technology, MPD instrumentation, and MPD applications are disclosed in the patents listed in the *Background* section and are incorporated herein in their entirety. The inventor has developed a family of MPD instruments. The SSMPD (sequential sample MPD) is a replacement for traditional radioimmunoassay counters and is used for super-sensitive quantitation of biological samples up to a few milliliters in volume. Sub-zeptomole sensitivity (better than 10^{-21} mole/ml) has been achieved. A 510k Pre-Market Notification from the FDA for the SSMPD and MPDATA for Windows (MPDATA) control and data logger software has been obtained and is commercially available for use in clinical laboratories. The planar geometry of the MPD detector has enabled the development of SRMPD (spatially resolving MPD) for the concurrent measurement of multiple samples. The current SRMPD processes up to 225 samples with zeptomole sensitivity. It has been used to quantitate radiolabel in dot blots and microwells with femtoCurie sensitivity. The SRMPD is capable of measuring samples concurrently in an industry-standard microtiter plate. We developed the SR-MPD/MT 96 and SR-MPD/MT384, which permit quantitation of 96 and 384 well plates with sub-zeptomole/well sensitivity. The cross-talk between the wells is below 0.1 percent. Note that the same detectors can be used for quantitation of microtiter plates and membranes with the same pattern of pixels as the wells in microtiter plates. The MPD/MT 1516 has been designed and will be available in summer 2001.

MPD Imagers are suitable for analysis of two-dimensional sample formats such as gels, dot blots, DNA binding microarrays or protein binding microarrays, like the invention herein. We achieved a few zeptomole sensitivity for ^{125}I and 0.1 sensitivity is expected for ^{123}I. The MPD Imager is supported by proprietary imaging software, *Laner* for Windows that is specifically designed for analysis of qPCR blots and gels. We have demonstrated experimentally that the MPD Imager system is at least a factor of 1000 more sensitive than a phosphor imager. The MPD Imager permits 0.006 attomoles of DNA per band versus Molecular Dynamics Phosphor Imager, 6 attomoles per band.

The inventor and the assignee of record (BioTraces, Inc.) have produced twenty MPD instruments, of which about ten are currently used in several biological laboratories including NIH, NIST, NASA, NeXstar and a few sites in Europe. The parameters for available MPD Imagers are shown in **Table 2**

Table 2: MPD Imagers available from BioTraces, Inc.

Model	Array Size	Size of Blot (cm x cm)	Background(cpm)	
			OR	AND
MPD-Im/121	11 x 11	20 x 18	0.03	0.0003
MPD-Im/225	15 x 15	20 x 18	0.02	0.0002
MPD-Im/900	4 x 15 x 15	20 x 18	0.02	0.0001
MPD-Im/1.6K	4 x 20 x 20	20 x 18	0.02	0.0001

16. The term "multi photon emitting radioioisotope" used herein refers to a radioiotope, which emits at least two detectable particles in the process of decay. These include the "electron capture" radioisotopes, *i.e.* alternate form of an element that has the usual number of protons but a smaller number of neutrons than the predominant stable isotope. Such isotopes tend to absorb electrons from the shell ("electron capture") which generally leads to emission of two high energy photons, typically X-ray and gamma-ray. The preferred radioisotopes used in the invention are ^{123}I, ^{125}I, and ^{32}P. Most preferably, ^{123}I and ^{125}I are implemented in reusable protein chip assays. The life time of ^{123}I is 13.2 hours. This is an electron capture (EC) isotope, which decays with coincident emission of X-ray from the shell (27 or 31 keV) and a gamma ray from the nucleus (159 keV). Life time of ^{125}I is 60 days. This is an electron capture (EC) isotope, which decays with coincident emission of X-ray from the shell (27 or 31 keV) and a gamma ray from the nucleus (35 keV).

The ^{123}I can be efficiently produced from ^{124}Xe. The isotope ^{124}Xe is available and can be efficiently used in its liquid state. The use of a liquefied inert gas as the target enables a clean and low cost radiochemistry in which the ^{123}I salted out of the liquid phase ($Na^{123}I$) and ^{124}Xe is recycled with minimal loss. The ^{123}I is available commercially with timely delivery, daily or about every 12 hours. Optionally, it can be produced on site using low cost, table-top accelerators to generate the strong beam of photons with the required energy of 100-200 keV *via* brehmsstrahlung.

Finally, the use of short life radioisotopes decreases on the problem of storage of mixed, biological and radioactive waste. After ^{123}I decays completely the resulting waste is purely biological.

Radiolabeling nucleic acids is a technique well known in the art and we propose the use of ^{123}I-dCTP. All biochemistry is the same as for ^{125}I-dCTP which is also available commercially.

Manufacture of reusable microarray

Preparation of analytes: A detailed description of analyte preparation, radiolabeling, and coincidence detection by multiphoton technology is disclosed in Drukier, et al., *Enhanced chromatography using multiphoton detection*, U.S. Pat. No. 5,854,084 and incorporated herein in its entirety.

First, a protein isolation step entails the use of appropriate extraction methods. Such methods entail the extraction of tissue or physiological fluid and centrifugation of the sample (i.e. Differential centrifugation or rate-zonal centrifugation). Generally, samples are eluted in lysis buffer such that cellular material can be separated from the protein of interest.

Second, to avoid assay binding of non-specific background the most abundant proteins must be removed. Physiological fluid contains more than 80% total mass of abundant proteins such as albumin, hemoglobin, and myoglobin. These proteins can be removed through isolation methods known in the art such as affinity chromatography or capture by magnetic beads coated with antibody specific to the respective abundant protein.

Third, because of the complications inherent in protein secondary structures (concealment of epitopes, ligand binding domains, enzyme reaction domains, *etc*...), protein denaturation alleviates such complications and leads to more reliable quantification. Since the invention also uses aptamers as target specific probes, protein denaturation may not be suitable for some assays. Nucleotide:protein interactions depend on intact binding domains for correct alignment. (Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P., Darnell, J. *Molecular Cell Biology*, 3rd edn., Scientific American Books. 1995)

Fourth, the collection of proteins in an analyte can be radiolabeled, preferably radioiodinated. Optionally, if a secondary labeling probe is used (i.e. sandwich assay, or radioiodinated streptavidin) proteins are biotinylated.

Analysis of analyte

Target proteins from the analyte are immobilized by direct application to the microarray surface where probe molecules have been anchored. All target proteins are captured in parallel and the anchored antibody or aptamer probes are present in excess of the proteins from the analyte (greater than 1000 fold, preferably 10,000 fold, and most preferably 100,000 fold). Given the varying concentrations of some proteins in an analyte it is useful to have pixels of varying surface area in the microarray matrix. Optionally, one can allocated a portion of the microarray matrix to probe for a specific target protein (e.g. 2x2, 3x3, 4x4, or 5x5).

Upon analyte application, a washing step is necessary to rid the assay of unbound or weakly bound proteins. Preferably, the washing step is to be repeated several times.

The washed microarray having target proteins bound specifically to their appropriate probe molecules and spatially resolved along the surface of the array is exposed to the MPD imager for coincidence detection. The MPD Imager interfaces with a computer containing imaging software for display of the resolved emission patterns and corresponding said emissions to the position of the probe molecules. The level of coincidence corresponds directly and is proportional to the quantity of a specific target protein contained in the analyte applied to the microarray.

Since the probe molecules are present in great excess of the desired target proteins, a vast majority of the probe molecules will remain unbound and available for subsequent assaying. Furthermore, since the half life of ^{123}I is short (less than two days), the extinguished radioisotopes give rise to a "clean slate" and allow unambiguous subsequent radioassaying thus rendering the microarray reusable.

The P-chip vs. panel of immunoassays: The inventor points out that the P-chips are often using the same target specific moieties as panels of immunoassays. The inventor defines P-chips as systems that detect preferably more than about 20 proteins in parallel. Obviously, one could put the border between the P-chips and panels of immunoassay on 50 proteins. There is, however, a good historical and technical argument, why 20 targets quantitated in parallel is appropriate. First, historically, panels of immunoassays targeting up to about 10 proteins have been used in immunodiagnostics. The best example are the panels for microbial pathogens. Other example is the typical panels used in "blood image" assessment. Second, in the framework of dP-chips, it is essential to quantitate all "physiologically important" proteins in a given family. For example we know about 20 chemokines, 20 interleukins, about 60 cytokines and about 20 natural antibodies for cancer. Finally, the main argument considers the sensitivity. In majority of cases, *e.g.* needle biopsy the amount of sample is very small, say less than 100 microliters. The sensitivity of individual immunoassay is always higher than the sensitivity of P-chip. Typically the loss of sensitivity by factor 20-50 as compared with best immunoassay is to be expected. Thus, the sensitivity of panel of immunoassays is better when there is less than 20 targets in the panel. Similarly, for diagnostics one typically takes about 1 ml of blood. The typical immunoassay in a 96 well microtiter plate takes about 100 microliters. Thus, 1 ml of blood can be aliquoted into 10 samples. When using 384 well microtiter plates, one uses about 50 microliter per well. Thus, when using 1 ml of blood, 20 aliquots can be used to measure up to 20 targets in a panel of immunoassays. We note, that even if IA is sensitive enough, the further diminishment of sample volume leads to artifacts due to non-specific biological losses.

The economics of P-chips: There are essentially four markets for P-chips:

* massive preventive diagnostics (market of 10-20 billion dollars);
* therapy monitoring (market of 1 billion dollars);
* pharmacoproteomics, including drug validation tests (market of 0.5 billion dollars);
* life science applications (market of 0.25 billion dollars)

However, each of these markets requires a different type of P-chip and sensitivity . For first two applications, the cost is extremely important. These characteristics are described in the Table 3.

Table 3: Different Markets for P-chips.

Market	Tests/y	Targets/chip	Sensitivity	Max. Price
Massive Diagnostics	10^9	1,000	1 pg/ml	$20/assay
Therapy monitoring	$5x10^7$	100	0.05 pg/ml	$50/assay
Pharmacoproteomics	10^6	500	0.1 pg/ml	$500/assay
Life Science	10^5	500	0.05 pg/ml	$500/assay

The current state of art in P-chips is that cost is about $1 per chip and the sensitivity is at about 50 pg/ml. Thus, both cost and sensitivity has to be improved by factor 50-100 to allow full penetration of the above said markets. This invention disclose how the cost challenge can be resolved by use of reusable P-chips. We also disclose, that MPD enhanced chips can eliminate the non-specific biological background and allow the P-chips with the sensitivity down to 50 fg/ml.

The low abundance proteins are the preferred target of P-chips: In the field of Proteomics three simple questions are asked:

1. What proteins are present in a biological sample?
2. How do these proteins change in response to disease?
3. Which of these proteins can be exploited for diagnostic or therapeutic purposes?

Proteomics is both complementary to and extension of functional genomics. Modern methods of genomic analysis assay the gene activity using DNA microarray technology to measure the presence of specific mRNA molecules. Unfortunately, knowledge of mRNA levels does not accurately reflect the presence or activity of the corresponding protein molecules. Proteins are often modified after they are synthesized, and these modifications are often the actual determinants of activity. Thus attention is increasingly being focussed not on the nucleic acids but on the proteins. Thus proteomics, the study of the proteome, is recognized as being necessary for the next decade of biomedical studies. With the harvesting of biological information resulting from such studies, new levels of understanding of biological and pathological processes will be reached. Importantly, these new levels of understanding will enable rational intervention in a vast variety of economically important cases, including disease

treatments. Thus, biomedical community is increasingly interested in biologically and functionally important molecules, *i.e.* proteins.

Diagnostic proteomics requires higher sensitivity analytical methods, because it is impossible to amplify proteins. A fundamental issue in proteomics is, and will be, the sensitivity of the methods to detect and analyze proteins, their modified isoforms and multi-subunit complexes. Current immunoassays and discovery proteomics technologies do not fulfil the high expectations of the various interest groups of the health care business because it could detect only high abundance proteins. Low abundance proteins are the molecules that shape the function and destiny of cells and tissues. They are often documented to be molecular switches, and are the most prominent targets and products for the pharmaceutical industry. Thus, it is necessary to dramatically improve the capability to analyze the proteome of cells and tissues. Therefore successful entrants into this field will have to focus on the emerging techniques for detection, quantitation and understanding properties of sub-attomole/ml proteins. We note that typical estimates talk of about 100,000 unknown proteins. It is plausible that the successful proteomics techniques will specialize in specific sub-fields of proteomics (discovery *vs.* diagnostic) stratified either by type of disease or by the sub-group of proteins. For example, due to its superior sensitivity of the P-chips disclosed herein, we will be able to detect more functionally important low abundance proteins than can be detected by *current* methodologies.

It is cheaper to make low cost P-chips if they are targeting low abundance proteins:

As to the economics of the diagnostic assays using P-chips: Generally, the cost of such diagnostic assay can be divided onto three groups of costs:

* sample acquisition and assay performance;
* cost of production of P-chip;
* cost of data analysis.

The assays can be divided into two groups: using physiologic fluids, *e.g.* blood or urine and using clinical samples. In the first case, the physiologic fluids are typically obtained for all diagnostics, not only diagnostics based on P-chips. The cost is low, around $10, the sample can be easily aliquoted between a plurality of experiments and cost of storage is low. There are procedures, when the samples of a particular tissue can be obtained rather easily, *e.g.* PAP

smear, laryngological samples, skin samples. Also in this case, the cost of obtaining a sample is not dominating. However, for many tissues microsurgery, *e.g.* needle puncture is required. The act is performed only rarely and the cost is high because can not be performed in ambulatory setting. When measuring the clinical samples of this nature, the cost of the assay is less important consideration but the use of most sensitive assay is crucial.

Cost of the diagnostics act can be divided into:

* selection and production of target specific moieties, e.g. antibodies;
* production of the P-chip;
* storage of the P-chip;
* performance of the assay using already available biological sample.

Currently, the selection and production of target specific moieties is responsible for a major fraction of the cost of diagnostics based on P-chips; we estimate this fraction to be between 50-80%. This is why many groups works on methods permitting mass production of "libraries of target specific moieties", *e.g.* phage grown antibodies, or aptamers. However, Abs produced by classical hybridoma techniques still shows some considerable advantage in sensitivity over other techniques. The classical production methods has , however two main limitations: cost and batch-to-batch irreproducibility. Concerning the cost of Abs, from a single animal, say mouse, one can obtain a few microgram of a particular Ab. With a molecular weight of about 100,000 Dalton, this is equivalent to about 10 picoMole of the Ab. When trying to detect medium abundant proteins, the level of target is at about femtomole/sample. The amount of Ab should be in at least 10,000 fold excess. Thus, one needs about 1 picoMole of Ab for each such target. Thus, from a single batch of Ab, one can produce about 100 and 1,000 P-chips targeting HAP's and MAPs, respectively. With a cost of a batch of Abs at about $1,000/batch, the cost of Abs per a single P-chip is about one dollar per target.

Note also, that each batch of Abs is different. When HAPs and MAPs are targeted, the maximal size of batch of P-chips is about hundred. Herein, by a batch of P-chips, we understand a batch of P-chips produced from the same Abs. Thus, every 100 batch produced, must be tested and calibrated, which additionally increase the cost of P-chips.

The application of P-chips to low abundance proteins eliminates the above said limitations. The LAPs are present in biological fluids at less than about 10 attomole/ml level. Thus, a particular batch of Abs can be aliquoted to produce about 100,000 P-chips, which eliminates the problem of batch-to-batch reproducibility. Furthermore, the cost of Ab per target become a few cents per target protein, *i.e.* is negligible for diagnostic P-chips featuring about 100 target proteins. It become acceptable even for larger screening P-chips; with about 1,000 targets per chip a total cost of Abs is about 10-20 dollars. However, the construction of P-chips targeting the LAPs requires two changes:

* use of "sandwich" type of P-chips to eliminate the NSBB;
* use of more sensitive detection techniques, *e.g.* MPD to quantitate the amount of the LAPs.

Both of these innovative concepts are disclosed in this patent.

Currently, the cost of the placement of Abs on the surface of P-chip is very high, about 50 cents per target or about 100 dollars per chip featuring thousand targets. For a current generation of P-chips this cost is comparable to a cost of used Abs. However, when targeting LAPs and using more sensitive detectors, *ergo* diminishing cost of antibodies tenfold, the "place on the chip" costs will dominate. This is a main rational for the reusable "P-chips" disclosed in this patent.

The storage of produced microtiter plates is a non negligible fraction of cost of immunoassays and panels of immunoassays. However, P-chips are about 100 times more dense, i.e. the cost of storage of P-chips is negligible.

Finally, the cost of analyzing the results of P-chip are currently very high. The analysis is not yet automated and requires the participation of highly trained personnel. Assuming about one hour per screening P-chip, this cost currently dominates the cost of P-chip based diagnostics. However, this step can be clearly automated. We believe that the further development of bioinformatics tools will permit high throughput/low cost analysis of patterns obtained by even largest P-chips. The techniques will allow the most reliable, most sensitive P-chips and new methods of bioinformatics are being developed and will be disclosed elsewhere.

Mixed moiety P-chip: The analysis of the costs of Abs necessary to create the P-chips suggests a new, innovative concept/. Currently, all groups working on P-chips assume the "one source"

P-chips, *i.e.* assume that all target specific moieties are of the same nature, classically grown Abs or phage display Abs or aptamers. We disclosed a concept of "mixed" P-chips, wherein the HAPs and MAPs are targeted with low cost/lower specificity target specific moieties, *e.g.* phage display Abs or aptamers. The Laps, however, are targeted with high cost but best available specificity Abs.

Currently, the P-chips designers assume that the size of all pixels is the same. This considerably facilitates the production and diminishes the cost of the P-chips. However, we should point out that only a limited density of antibodies can be attached to a pixel of a given surface. Typically, one can attach about a femtomole of Ab per a pixel of square millimeter. Thus, the size of pixels targeting HAPs should be about 100 times larger than size of pixels targeting MAPs, whereas the pixels targeting LAPs may be sub-millimetric.

Finally, we should address the issue of nonspecific biologic backgrounds. Herein there is a clear trade-off. The ten most abundant proteins, mainly albumin and immunoglobulins account for 90% of mass of proteins in blood and, in our estimates, accounts for comparable fraction of non-specific biological background. Thus, it is preferable to remove these proteins at the first step of analysis by P-chip. It can be achieved by dividing the P-chips into two zones dedicated to detection of HAPs and MAPs/LAPs, respectively. In particular application, the proteins from physiological fluid are first placed on a part of a chip when Abs to HAPs are present, the HAPs are captured and then fluid is moved toward the second part of the chip. Note, that even if the number of HAPs is only a very small fraction of total number of proteins, their abundance is much larger. Thus, the larger pixels have to be used, and the surface of the part of the P-chip dedicated to detection of HAPs may be larger than 50% of the total chip surface.

Other consideration is the non-specific biologic losses (NSBL). These losses are well calibratable, but the situation is different for MAPs and LAPS. In the case of MAPs the NSBL leads to need of recalibration, but do not influence the delectability. In the case of LAPs, *i.e.* proteins with a few attomole/ml abundance, the NSBL may move the LAPS below the level of delectability. Thus, it may be necessary to split the P-chip further into parts dedicated to MAPs and LAPS, respectively.

We described herein the mixed P-chip, wherein the chip itself is divided into three parts dedicated to detection of HAPs, MAPs and LAPS. Within each part, different type of target

specific moieties may be used, with Abs detecting LAPS. Furthermore, the pixel size can be different in each of the P-chip parts, with LAPs requiring the smallest size pixels.

Finally, there is a clear trade-off between methods for removal NSBB and NSBL. To eliminate the NSBB, the physiological fluid should flow first to a part dedicated to detection of HAPs, continue to part detecting MAPs, and only at the end will be for a longer time be allowed to be present in LAPs zone. However, to eliminate NSBL, the fluid should first be presented to LAPs detecting zone, will flow to MAPs zone and reach HAPs zone at the end. Alas, currently it is not clear whether NSBB or NSBL limits the performance of P-chips. Thus, the P-chip contains both geometries, i.e. six zones are present. In one half of the P-chip, the fluid motion is HAPs zone=> MAPs zone =>LAP zone. In the second part of the P-chip, the flow is reversed; fluid movement is LAPs zone+. MAps zone=> HAPs zone. After the measurement, the results from both flow directions are compared permitting elucidation of the effects of NSBB and NSBL.

The solution - MPD enhanced P-chips: In the above we noted, that the P-chips targeting low abundance proteins will need to have different level of sensitivity and modified structure, as compared with P-chips targeting only HAPs. The new generation of P-chips requires better methods of detection. We documented that MPD permits the development of IA/MPD which for end point immunoassays achieved the landmark 1 fg/ml or 20 zeptomole/target sensitivity. Multi photon detection is ideal for quantitating P-chips because of the superior properties of MPD, namely:

* zeptomole/sample sensitivity;
* excellent spatial resolution;
* superior reproducibility and immunity to biological matrix artifacts;
* ability to achieve multilabel read-out;
* a very large dynamic range.

The MPD technique and its advantages in P-chips production:

Multiphoton Detection (MPD) technology is the means by which target proteins at low abundance are quantitated with high sensitivity. Current methods can only resolve up to 10% of all proteins in given cell lysates or in physiological fluid and have diminished chance of detecting the critical molecules involved in a particular process. Improving the levels of

differential detection and efficient analysis from the present 5-10% to 50% of proteins in a reliable and robust manner would be a significant advantage over current methods. The disclosed invention increases the detection efficiency to 60-80% of differentially displayed proteins and enables detection of the majority of the most important molecules for a given disease. The use of these methods will permit detecting and characterizing the low abundance proteins that serve as the "molecular switches" in cellular function.

MPD technology relies on the ability to distinguish the highly specific decay signature of certain radioisotopes from the various forms of naturally occurring background radiation. By using patented methods to analyze each detected event, MPD distinguishes desired events from the overall background. As a result, MPD reduces the measurement background to less than one event per day. The isotopes compatible with MPD include over 100 members appropriate for use as reporter labels. In particular, they include ^{125}I. Because each isotope emits decay photons with different characteristic energies, MPD can identify and distinguish among several different isotopes in the same sample. It is this multicolor capability that permits simultaneous testing for several different analytes within the same sample and the inclusion of mobility or molecular size standards during fractionation procedures. Methods of radiolabeling, including radioiodination, are well known in the art.

Patents relating to MPD technology, MPD implementation, manufacturing of MPD devices, and related methods used in the present invention are disclosed in the following U.S. patent applications and are incorporated herein by reference in their entirety: U.S. Patent No. 5,083,026 entitled *Method, Apparatus and Applications of the Quantitation of Multiple Gamma-Photon Producing Isotopes With Increased Sensitivity,* issued January 21, 1992. U.S. Patent No. 5,532,122 entitled Quantitation of Gamma and X-ray Emitting Isotopes, issued July 2, 1996. U.S. Patent Application 08/669,970 entitled *Ultralow Background Mutiple Photon Detector,* filed June 25, 1996. U.S. Patent Application 08/679,671 entitled *Enhanced Chromatography Using Multiphoton Detection,* filed July 12, 1996.

Reusable P-chips: The need for reliable placing on a chip a few thousand capture agents (antibodies, aptamers) with a high reproducibility and precision is the main technical challenge of any effort in development of P-chips. Thus, automatic "bio-matons" are used featuring the array of pins to gently place droplets of appropriate buffer containing Abs on the surface of protein chip. All traditional processes use a relatively long process to incubate/bind the Abs to

the chip surface. Because of finicky nature of Abs these processes take a considerable time. The devices are expensive and thus with relatively low throughput, the cost of P-chip fabrication is a dominant part of the cost of diagnostic proteomics. Another important part of the cost is need for a non-negligible amount of expensive antibodies. This cost liabilities of use of antibodies means that the use of structures such as aptamers may be cost-wise an important advantage. However, we doubt that even with aptamers P-chips featuring 5,000 different capture moieties can be implemented for say less than $10 per chip. The margins will be slim but only the huge number of expected tests make the concept viable commercially.

For reusable P-chips, assuming 50 samples can be measured sequentially using the same P-chip, and cost per test of $20, the accumulated revenue per P-chip is $500-1000. With the cost of producing a P-chip about $50-100, this provides significant profits.

Diagnostic P-chips: We expect that between 10 to 20% of assays with screening P-chips will provide the information that "something is wrong" with a particular individual at a particular time. Actually, with the help of bioinformatics, the sP-chips may provide even more specific information. The sP-chips should be able to stratify the tested individuals into large sub-groups, and will suggest the need for advance check for microbial contamination, cancer, neurodegenerative disease or autoimmune disease. Thus, the fast development of sP-chip will generate a large market for more sensitive diagnostic P-chips (dP-chips). We estimate that the market for diagnostic P-chips is 10-20 million tests per year. Herein, however the sensitivity and reliability are essential and cost of lesser importance. We expect that such dP-chips will be economically viable at 100 dollars per assay. Thus, the modality of application of dP-chips has an estimated market of about a billion dollars per year.

There is however another application of the same dP-chips, namely, application in therapy monitoring. We estimate that this modality will be applied to about 5 million patients by the year 2005. With a test every month, this leads to 60 million tests/year, or a few billion dollar market. We note that this is probably the most profitable part of the diagnostic proteomics market. The cost of manufacturing and testing a dP-chip is expected to be below 10 dollars with sale price of about 200 dollars.

EXAMPLES

The following examples are solely for purposes of illustration and in no way limit the scope of the invention:

In the following we first describe the general aspects of creation of P-chips and the basic thermodynamics permitting the construction of reusable P-chips. We disclose three types of P-chips:

1) P-chips using antibodies as capture moieties;
2) P-chips using aptamers as capture elements;
3) "sandwich type" P-chips, wherein the proteins are bound to a surface of a given pixel by the capture antibody (Ab1) and the label is attached by another antibody (Ab2).

Concerning the "sandwich type" p-chips, the two antibodies should target the different epitopes on the protein; such a pair of antibodies is called "matched antibodies". Furthermore, the aptamers technique can be used in construction of sandwich type P-chips. Alas, typically it is difficult to evolve the matched pair of aptamers, *i.e.* aptamers binding to two different epitopes on the same protein. However, we disclose the two types of "mixed sandwich" techniques wherein:

* the aptamer is used to capture the protein and labeled antibody is used to label this complex, *i.e.* Ap-P-Ab sandwich;
* the antibody is used to capture the protein and labeled aptamer is used to label the complex, *i.e.* Ab-P-Ap sandwich.

We disclose the three new techniques which permit the implementation of reusable protein binding microarrays, *i.e.* reusable P-chips (rP-chips). These implementations use independent principles:

(a) use of direct radiolabeling with a very short lived radioisotope;

(b)use of labeling with specially constructed nucleic acid constructs and a step of enzymatic cleavage;

(c) use of labeling with specially constructed nucleic acids and a step of deactivating the label.

In general, the techniques used to "erase" the signal are similar to the ones disclosed for implementation of reusable DNA-chips. However, considerable modifications are also disclosed. Generally, the process of radiolabeling, especially radioiodination of proteins is simpler than radiolabeling of DNA. Thus, the implementation of techniques (b) and (c) requires attaching DNA constructs to the proteins, which is not easy.

Conventions: In the following, we will use the hybrid methods wherein the immunologic reagents (Abs, Aps) and streptavidin are coupled with appropriate DNA constructs. All conventions used in Phase I of this patent will be also used herein.

1. The reusable P-chips in simple binding format

Protein binding microarrays (P-chips) can be divided into two classes: screening P-chips (sP-chips) and diagnostic P-chips (dP-chips), which are quantitative. In the first case, a very large number of targets are measured, albeit with the limited sensitivity of a few pg/ml. The cost of such chips is extremely important. The diagnostic P-chips measure a smaller number of target proteins, about 100, but the sensitivity needs to be about hundred times higher, about 10 fg/ml. The cost of such chips may be about $50.

The sensitivity and economic constraints require that two different techniques be used, namely, a simple binding assay format for sP-chips, and a sandwich assay format for dP-chips. Generally, due to the very large number of pixels, the economic advantages due to development of reusable screening P-chips are higher. Thus, we first disclose the methods allowing implementation of reusable sP-chips.

We first describe the main steps of non-reusable P-chip technology. The label can be a radioisotope, a fluor or enzyme such as horseradish peroxidase. The detailed steps for implementation using radiolabeling, *e.g.* with ^{125}I are :

a. ***Protein isolation:*** In the case of proteins, especially membrane proteins, the use of appropriate extraction procedures is important. In many cases, the extraction method leads to denaturation of the proteins. It is important to both separate all proteins but, preferably, remove the natural polymerases and DNAses. This may be easier when physiological fluids, rather than cell extracts, are used.

b. ***Removal of the most abundant proteins:*** In the case of many biological samples, there is a very great range in concentration for the different classes of proteins. This is especially important in the case of physiological fluids. For example albumin and hemoglobin account for more than 80% of total mass of protein in blood. To diminish the non-specific biological background, the most abundant proteins are preferably removed, *e.g.* by affinity chromatography or capture on antibodies coated magnetic beads.

c. ***Protein denaturation.*** Because of complications induced by the secondary structure of proteins, the use of denatured proteins may lead to more reliable quantitation. However, when aptamers are used as a target specific moiety, the proteins should not be denatured.

d. ***Proteins labeling:*** The collection of pre-processed proteins can be radiolabeled, *e.g.* radioiodinated. The standard methods of radioiodination via Hunter -Bolton or iodobeads methods are adequate. The effort should be made not to over iodinate.

Optionally, the preprocessed collection of proteins can be biotinylated using standard procedures.

e. ***Protein capture:*** In P-chips, the immobilization is by spatially resolved binding to an immobilized library of antibodies (Abs) or aptamers (Aps). Standard protein and nucleic acid chemistry methods offer the possibility of making an array of capture probes and determining the abundance of all the probes, *i.e.* amount of antibodies/aptamers in each pixel. All proteins are captured in parallel and sufficient time is allowed so that almost all proteins are captured. This requires that the number of capture Abs/Aps at each pixel is much higher than the expected abundance of target proteins. Taking into account, the large range in concentration of proteins, it may be useful to have pixels of different surface. Optionally, groups of pixels say 2x2, 3x3, 4x4 or 5x5 will be dedicated to a given protein in proportion to their abundance. The excess of capture Abs/Aps should be 10,000 to 100,000 fold, *i.e.* about nanomoles of Abs/Aps is present on each pixel.

*f. **Stringent wash:*** Note that this step is absolutely crucial in the case of proteins . Actually, our practice shows that the wash procedure should be repeated at least three times to achieve the best possible sensitivity. Special washing reagents and procedures has been developed.

*g. **Counting and analysis:*** The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter will introduce essentially no error to the DNA quantitation.

There is a considerable advantage of using the radioisotopes and sensitive, quantitating imager such as MPD-Imager. First, the intrinsic signal/background is above 1,000 when quantitating femtomoles of proteins, and the measurement is limited mainly by statistical uncertainty. The number of detected counts in each pixel is high - typically we measure 400 counts from each pixel. Thus the uncertainty of the measurement is only 5%. Let assume a simple method in which a P-chip is measured shortly before the analysis of unknown protein sample. The activity at each pixel is measured and stored as look-up table in computer. Then the radiolabeled protein sample is hybridized, washed and the P-chip is re-measured. The abundance of each of the protein targets can be obtained by subtractive analysis using a simple computer calculation. Thus, taking in account excellent sensitivity, high reproducibility and high dynamic range of MPD, even when using long life time radioisotopes such as ^{125}I, we expect to be able to use the same P-chip a few times in less demanding screening applications.

Each step in the method has various alternatives, some of which are described in current literature. The screening P-chips are becoming an important, but not very sensitive, tool enabling parallel detection of the presence of hundreds to thousands of target proteins. However, prior art SP-chips can be used only for proteins with concentrations greater than a few femtomole per ml. Process optimization is crucial to achieve reliable quantitation of selected sub-sets of proteins. The use of ^{125}I and MPD permits increased sensitivity in simple binding P-chips by a factor of a few when compared with prior art.

II.1 The reusable P-chip using short life-time isotopes, including ^{123}I

The first disclosed innovation is the ability to create reusable P-chips by replacing ^{125}I with a short half-life radioisotope. There are about fifty short-life time isotopes, say $t_{0.5} < 2$ days which are compatible with supersensitive MPD based methods. Actually, the use of short-life time isotopes increases the sensitivity of radioimaging, and classical radio-imagers such as phosphor plate or autoradiography can be used. However, use of MPD imagers permits a few hundreds time higher sensitivity then phosphor imager.

The use of short life-time isotopes brings two advantages. First, the counting statistics are improved. Lets consider a radio-isotope with life-life of 12 hours and reasonable efficiency of labeling, say a single radioisotope atom per molecule. At 1 femtomole/pixel the decay rate is about 6,000 dpm. With a detection probability of 10% and a measurement time of 15 minutes per pixel, the expected count total is about 10,000 counts. Thus, the statistical uncertainty is about 1%. Thus using the method described above, the sequential quantitation of about 20 samples seems possible.

A second advantage of using short-lived isotopes as labels is that such a DNA-chip becomes self-erasing, *i.e.* the activity of the chip decays exponentially. If a radioisotope with a half life of 12 hours is used, the activity is diminished by factor 16 after 48 hours and factor 64 after 72 hours. Thus, such a self-erasing P-chip can be used about 100 times over a one year period.

We disclose the use of ^{123}I as an almost perfect isotope for the implementation of re-usable DNA chips. The half-life of ^{123}I is 13.xx hours. This is an electron capture (EC) isotope, which decays with coincident emission of X-ray from the shell (27 or 31 keV) and a gamma ray from the nucleus (150 keV). The ^{123}I is available commercially with "on time" delivery, say daily or even every 12 hours.

Fortunately, the radiolabeling of proteins is a well known technique. For the longer lived ^{125}I, we prefer the use of the Bolton-Hunter reagent. However, for the shorter lived ^{123}I, the production of $Na^{123}I$ is simpler and we propose the use of the iodobeads method. Importantly, the use of any short life-time radioisotope solves the problem of storage of mixed, biological + radioactive, waste. After a few days all the ^{123}I decays and the waste can be treated as pure biologicals.

We disclose the use of re-usable P-chips labeled with ^{123}I. The ^{123}I is an EC emitter. Actually, the X-rays are the same as for ^{125}I only gamma has higher energy (150 keV *vs.* 35 keV for ^{125}I). Thus it can be detected with SR-MPD/MT 96 and SR-MPD/MT 384 as well as with MPD-Imagers. We estimated that the sensitivity of MPD detector is better than 100 atoms of ^{123}I per pixel. The calculations in section 3.1 shows the expected count-rates. Thus P-chip using ^{123}I labeling and MPD imager can be used to quantitate about 100 samples/year.

Our invention is not limited to ^{123}I. There are about fifty short-life time isotopes, say $t_{0.5} < 2$ days which are compatible with supersensitive MPD based methods. Actually, the use of short-life time isotopes increases the sensitivity of radioimaging, and classical radio-imagers such as the phosphor plate or autoradiography can be used. However, the use of MPD Imagers permits a few hundreds time higher sensitivity then phosphor imagers.

The procedure is as follows:

a. ***Protein isolation;***

b. ***Removal of the most abundant proteins;***

c. ***Protein denaturation;***

d. ***Protein radioiodination:*** The collection of pre-processed proteins will be labeled with ^{123}I. Oxidative iodination methods will be used, and an effort should be made not to over iodinate.

e. ***Protein capture;***

f. ***Stringent wash;***

g. ***Counting and analysis:*** The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter reduces error to DNA quantitation.

II.2. Another implementation of reusable P-chips using ^{123}I

The first disclosed innovation is the ability to create the reusable P-chip by use of ^{123}I and direct radiolabeling of the proteins. This method may require a radioactive license. An innovative

implementation of reusable P-chip involves the step of protein biotinylation and subsequent use of ^{123}I-streptavidin. The ^{123}I-streptavidin will be available for "on time" delivery twice a day.

The main advantage is that only one radioiodinated reagent is used. The potential liability is that streptavidin is sticky, *i.e.* the non-specific biological backgrounds (NSBB) may be somewhat higher than when directly radioiodinating the proteins. Thus, the steps of stringent washing and use of appropriate blockers are crucial for this implementation.

The procedure is as follows:

a. Protein isolation;

b. Removal of the most abundant proteins;

c. Protein denaturation;

d. Protein biotinylation: The collection of pre-processed proteins will be labeled with biotin. Standard methods will be used, and an effort should be made not to place more than one biotin per protein.

e. Protein capture:

f. Stringent wash:

g. Blocking: Because of the rather high stickiness of Streptavidin to plastic, the use of special blocking procedures is necessary. These permit a decrease in the binding of ^{123}I-streptavidin to the plastic by about five-fold.

h. Stringent wash;

i. The labeling via biotin-^{123}I streptavidin coupling: This is a classical technique used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

j. Counting and analysis: The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter will introduce essentially no error to the DNA quantitation.

k. Storage in dialyzing conditions. The capture moieties (Abs and less Aps) are very fragile and easily lose their specificity when stored. The presence of ^{123}I leads to additional challenges- the decay leads to creation of free radicals which may interact with Abs. Thus, it is necessary to store P-chips in an appropriate environment. The classical method is to dry the

P-chips and store in low temperature (but not below the freezing point of water). The innovative method is storage in specially prepared dialysis chamber, wherein the free radicals are permanently removed and/or trapped by antioxidant reagents.

II.3 Reusable P-chip with the step of cleavage by restriction enzyme(s) and ^{125}I:

One method for reusable P-chips involves a special construction of the labeling probe. Part of the construct is a <switch> element that can be cleaved enzymatically. The preferred implementation uses double stranded DNA and a sequence recognized by an appropriate restriction enzyme as a <Switch> and has been disclosed above in section I.3.3. In all disclosed implementations, the <switch> can be cleaved only when attached to a streptavidin. The streptavidin is used to interact with biotinylated proteins. Actually, in all of these implementations we can select from a large family of switches because there are a few hundred restriction enzymes (see I.3.3 for a list of the restriction enzymes which are specific for 6-mers).

One embodiment employs a <switch>, a complex of streptavidin with a DNA construct. Actually, there are three possible constructs of this type.

[<DNA complex>

labeling complex /1,3/> =]<streptavidin>[<DNA complex>

[<DNA complex>>

[<DNA complex>

<labeling complex /2,2/> =]<streptavidin>[

[<DNA complex>

]

<labeling complex /3,1/> =] <streptavidin>[<DNA complex>

]

Here the symbol "] " stands for an unoccupied binding site on streptavidin. When it is occupied by biotin, the interaction is denoted as " [< ". Thus, <labeling complex/3/1> describes streptavidin with three binding sites free, and one center occupied, *i.e.* to which the DNA complex is attached *via* a biotinylated linker. In the following, we will assume that the <labeling complex /3/1> is used, but this case can be easily generalized to other two complexes.

<DNA complex> = <linker><switch><radiolabeled-terminal>

Herein <linker> is a 20-30 mer dsDNA biotinylated at the 3-prime end. Switch is a 6-mer site for an appropriate restriction enzyme. Radiolabeled terminal is an arbitrary length dsDNA which is radiolabeled *via* ^{125}I-dCTP.

The simplest, but somewhat expensive method, is to produce the <DNA complex> on a DNA synthesizer using non-modified nucleotides, biotinylated nucleotides and radiolabeled nucleotides.

We disclose a preferred structure for the <DNA complex> that allows low cost production. The disclosed structure of such a *<DNA complex>* is as follows:

<DNA complex> = <biotinylated AT linker><AT switch>< radiolabeled terminal>

Here both the <biotinylated AT linker> and the <AT switch> contain no C or G bases, whereas <radiolabeled terminal> is CG rich. The two available <AT switches> are Asn I [ATTAAT] and Dra I [TTTAAA]. First the dsDNA without use of biotinylated nucleotides or ^{125}I-nucleotides is

produced on the synthesizer. Then the PCR in presence of biotinylated primers and ^{125}I-dCTP is performed.

The overall process of analysis using such "enzymatic cleavage" based reusable DNA-chip is as follow:

*a. **Protein isolation;***

*b. **Removal of the most abundant and of all naturally biotinylated proteins;***

*c. **Protein biotinylation:*** The collection of pre-processed proteins will be labeled with biotin. Standard methods will be used, and an effort should be made not to place more than one biotin per protein.

*d. **Protein capture;***

*e. **Stringent wash:***

*f. **Blocking:*** Special blocking procedures are necessary because of the rather high stickiness of Streptavidin to plastic. These permit about a five-fold decrease in the binding of ^{123}I-streptavidin to plastic.

*g. **Stringent wash;***

*h. **The labeling via <labeling complex>:*** This is a classical technique used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

*i. **Counting and analysis:*** The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter will introduce essentially no error to the DNA quantitation.

*j. **Cleavage using a restriction enzyme.***

*k. **Stringent wash and storage:*** Note, that in this implementation, no radioactive label is left after the cleavage step. Thus, the storage may be easier than in two first methods implementing reusable P-chips.

A preferred implementation uses restriction enzymes for which the restriction enzyme site consists of only A and T bases. We disclose the use of highly selective restriction enzymes Asn

I [ATTAAT] and Dra I [TTTAAA]; the restriction enzyme site consisting of a six nucleotide pattern is shown in brackets.

Note that this implementation allows us to operate with P-chips that are not radioactive, and then to perform the step of labeling via a universal radiolabeled streptavidin based complex. Thus, there is no issue of storage of prepared DNA-chips and use of radioactivity is limited to a short time during counting. Optionally, the sample DNA can be labeled with fluorescent or enzymatic labels, but then the sensitivity of detection will be much lower.

Example of Implementation: In this example we implemented the hybrid <Streptavidin><dsDNA>, wherein the structure of the <biotinylated dsDNa is as described in the patent. We documented that introduction of a single restriction enzyme sites permits 80% cleavage, 2 different restriction enzyme sites permits 90% cleavage and by incorporating a three different restriction enzyme sites, we achieved the cutting probablility of better than 95%.

PCR was done on 8 tubes with bound primer for the Listeria mimic and primer pG3 in solution. Product was denatured with NaOH to leave one bound strand.

Primer Bio-pG3 was tailed with I^{125}-CTP and hydridized to all eight tubes (column 1, bound primer).

The bound primer in all eight tubes was extended by a single PCR cycle of 40C, 5 min, 70C, 10 min (column 2, PCR).

Neutravidin was bound to wells 1-4 while wells 5-8 were incubated in same buffer. The bound DNA was digested with Not I and the DPM in supernatant measured (column 3, Not I sup).

The supernatants were added to biotin tubes (column 4, biotin plate). The avidin sups bound 48 DPM, the control sups bound 12 DPM.

Table 3

1		4980		1263		802		44	
2		4987		1220		816		54	
3		4930		1219		848		39	
4		4738		1188		890		54	47.75
5		4268		1132		885		13	
6		4723		1138		772		16	
7		5220		1262		872		9	
8		4939		1420		958		11	12.25

Column 1: DPM in PCR product

Column 2: DPM cut w Not I, tubes 1-6 had Neutravidin, 7,8 did not (control)

Column 3: DPM bound to biotin plate

Column 4: DPM cut from biotin, only used 4 tubes (tubes 1 &2), control no restriction enzyme (3&4)

1	3664	1665	94	14
2	4074	4909	317	42
3	4104	4085	241	149
4	4483	2957	148	92
5	4063	2442	2206	
6	4805	2512	165	
7	4162	3427	21	
8	4608	4377	16	

II.4 Reusable P-chips with the step of fluor-quenching.

We disclose an implementation of a reusable P-chip that is compatible with fluorescent labeling. We use the labeling complex:

]

< fl-labeling complex /3/1> =]<streptavidin>[<fl-terminal>

]

Herein, the <fl-terminal> is an arbitrary length dsDNA which is biotinylated and is labeled with a quenchable fluorescent label. We will also use a universal <fluor label quencher> also called <fl-quencher>, *i.e.* an oligonucleotide complementary to <fl-terminal> and able to quench the fluorescent label on <fl-terminal>.

Obviously, similar complexes <fl-labeling complex/2/2> and < fl-labeling complex/1/3> can be used as well.

The overall process of analysis for reusable P-chip based on activated/deactivated fluorescent labeling " is as follows:

a. ***Protein isolation;***

b. ***Removal of the most abundant and of all naturally biotinylated proteins;***

c. ***Protein biotinylation:*** The collection of pre-processed proteins will be labeled with biotin. Standard methods will be used, and an effort should be made not to place more than one biotin per protein.

d. ***Protein capture;***

e. ***Stringent wash;***

f. ***Blocking:*** Because of the rather high stickiness of Streptavidin to plastic, the use of special blocking procedures is necessary. It permits to diminish the capture of ^{123}I-streptavidin on plastic about five-fold

g. ***Stringent wash;***

h. ***The labeling via <labeling complex>:*** This is a classical technique used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

i. ***Counting and analysis:*** The samples can be counted in a standard microplate fluorometer.

j. The hybridization of <fl-quencher>:

k. Stringent wash and storage: Note that in this implementation, no radioactive label is used. Thus, the storage may be easier than in first two methods implementing reusable P-chips.

II.5 Reusable P-chips using resonant energy transfer.

This implementation is somewhat similar to II.4 but instead of a fluorescent label a resonant energy transfer mechanism is used. Furthermore, to quench the signal, the two strands of DNA in <RET terminal> are displaced with use of the peptide nucleic acid (PNA) leading to creation of PNA induced bubble. We note that the mechanism responsible for the reusability is creation of a PNA bubble, which has been shown to be very specific for properly selected DNA.

We disclose an implementation of reusable P-chip which is compatible with resonant energy transfer (RET) labeling. Herein, we use a labeling complex

]

< RET-labeling complex /3/1> =]<streptavidin>[<RET-terminal>

]

Herein, the <RET-terminal> is an arbitrary length dsDNA which is biotinylated and is labeled with a RET label. We will also use a PNA opener that leads to creation of a PNA bubble that blocks the resonant energy transfer.

Similar complexes <RET-labeling complex/2/2> and <RET-labeling complex/1/3> can be used as well.

The overall process of analysis for reusable P-chip based on active/deactivated RET labeling is as follow:

a. ***Protein isolation;***

b. ***Removal of the most abundant and of all naturally biotinylated proteins;***

c. ***Protein biotinylation:*** The collection of pre-processed proteins will be labeled with biotin. Standard methods will be used, and an effort should be made not to place more than one biotin per protein.

d. ***Protein capture;***

e. ***Stringent wash;***

f. ***Blocking:*** Because of the rather high stickiness of Streptavidin to plastic, the use of special blocking procedures is necessary. These decrease the binding of ^{123}I-streptavidin to plastic about five-fold

g. ***Stringent wash;***

h. ***The labeling via <RET labeling complex>:*** This is a classical techniques used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

i. ***Counting and analysis:*** The plates can be counted in a standard microplate fluorometer.

j. ***The hybridization of <PNA openers and creation of PNA bubble.***

k. ***Stringent wash and storage:*** Note, that in this implementation, no radioactive label is used. Thus, the storage may be easier than in two first methods implementing reusable P-chips.

II. 6 Use of combinations of disclosed techniques for reusable P-chips

Generally, each of the above disclosed methods is able to provide efficient mechanism for erasing the signal a short time after the measurement is accomplished. In some cases, however, say when more than 50 repeatedly performed measurements are required, it may be necessary to erase the signal by better than a factor of 100. In such a case, the synergic use of two mechanisms cutting the signal may be appropriate.

For example, when using the fluorescent or RET labeling, the <switch> can be introduced in front of <fl-terminal>. In this case, the procedure will be:

a. ***Protein isolation;***

b. ***Removal of the most abundant and biotinylated proteins;***

c. ***Protein biotinylation:*** The collection of pre-processed proteins will be labeled with biotin. Standard methods will be used, and an effort should be made not to place more than one biotin per protein.

d. ***Protein capture;***

e. ***Stringent wash;***

f. ***Blocking:*** Because of the rather high stickiness of Streptavidin to plastic, the use of special blocking procedures is necessary. It permits to diminish the capture of ^{123}I-streptavidin on plastic about five-fold

g. ***Stringent wash;***

h. ***The labeling via <labeling complex>:*** This is a classical techniques used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

i. ***Counting and analysis;***

j. ***Cleavage of <switch>;***

k. ***The hybridization of <fl-quencher>:***

l. ***Stringent wash and storage:*** Note, that in this implementation, no radioactive label is used. Thus, the storage may be easier than in two first methods implementing reusable P-chips.

Steps (j) and (k) can be performed concurrently.

7. Reusable diagnostic P-chips in "sandwich format"

We believe that due to the non-specific biological backgrounds, the simple capture P-chips have limited sensitivity. Currently, at the best, this sensitivity is about femtomole/ml. The

only exception may be the aptamer based simple capture P-chips when additional step of photolinking can be performed. However, even in this case we expect that sensitivity will be around 0.1 fmole/ml. However, in many biomedical applications the levels of important biologicals are much lower, say a few attomole/ml. Sub-attomole/ml sensitivity has been achieved in MPD enhanced immunoassays (IA/MPD). The generalization of a single IA/MPD to a diagnostic P-chip is possible. Most of the steps are *current* but some of the steps are innovative. They will be disclosed in another patent.

It will be economically important to implement the reusable diagnostic P-chip in sandwich format. To improve sensitivity, the two antibodies should target different epitopes on the protein; such a pair of antibodies is called "matched antibodies" and denoted Ab1(i) and Ab2(i), respectively. Ab1(i) are called capture antibodies and Ab2(i) are called labeling antibodies. Essentially, all methods described above can be used. For example, in sandwich assay Ab1(i)-P(i)-Ab2(i) we could radiolabeled with ^{123}I all Ab2(i). However, this will lead to a need of handling a very large number Ab2(i) each of short life. Thus the preferred implementations of reusable diagnostic P-chip are based on use of a single labeled reagent. This reagent is used to label all, appropriately derivatized , Ab2(i). Practically, the simplest implementation use the library of biotinylated Ab2(i) which is subsequently interacted with appropriate, universal streptavidin based labeling complex.

Aptamers can be used in sandwich type P-chips. Alas, typically it is difficult to evolve the matched pair of aptamers, *i.e.* aptamers binding to two different epitopes on the same protein. However, we disclose the two types of "mixed sandwich" techniques wherein:

* the aptamer is used to capture the protein and labeled antibody is used to label this complex, *i.e.* Ap(i)-P(i)-Ab(i) sandwich;
* the antibody is used to capture the protein and labeled aptamer is used to label the complex, *i.e.* Ab(i)-P(i)-Ap(i) sandwich.

For the sake of simplicity, in the following we disclose the preferred implementations of the reusable diagnostic P-chip in Ab1(i)-P(i)-Ab2(i) format. The extension to diagnostic P-chips using aptamers is straightforward.

I.7.1 The reusable diagnostic P-chip in "Ab1-P-Ab2 sandwich format" using ^{123}I and MPD.

We disclose a method for reusable diagnostic P-chips which involves the special <labeling complex> including a <switch> element which can be cleaved enzymatically. The library Ab2(i) is biotinylated and in labeling step is interacted with <labeling complex> via <streptavidin>.

The following description is for:

]

<^{123}I labeling complex /3,1/> =] <streptavidin>[<^{123}I-labeled DNA complex>

]

but use of <^{123}I-labeling complex /2,2/> and <^{123}I-labeling complex /1,3/> is also disclosed.

The overall process of analysis using such <^{123}I-labeling complex> based reusable DNA-chip is as follow:

a. ***Protein isolation;***

b. ***Protein purification:*** herein the removal of the most abundant, naturally biotinylated proteins and DNAses is performed.

c. ***Protein capture;***

d. ***Stringent wash***

e. Blocking: Because of possible stickiness of library AB2(i) to plastic, the use of special blocking procedures is necessary. It permits to diminish the NSBB about 5-fold.

f. Stringent wash;

g. Conjugation of library of biotinylated antibodies Ab2(i);

h. Stringent wash;

i. Blocking: This step is very important due to the stickiness of streptavidin. It typically permits a five fold improvement in sensitivity.

j. The labeling via <123I- labeling complex>;

k. Stringent wash;

l. Counting and analysis: The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter will introduce essentially no error to quantitation.

m. Storage: Note, that in this implementation, the short life time radioactive label is left to decay during storage. Thus a special storage conditions diminishing the radioactive damage should be used.

Note, that this implementation permits us to operate with P-chips which are not radioactive, and then perform the step of labeling *via* a universal radiolabeled streptavidin based complex.

II.7.2 Reusable diagnostic P-chip with the step of cleavage by restriction enzyme(s)

We disclose a method for reusable diagnostic P-chips in sandwich format which involves a specially constructed labeling probe. Part of the construct is a <switch> element that can be cleaved enzymatically.

The following description is for:

]

<^{125}I labeling complex /3,1/> =] <streptavidin>[<linker><switch><^{125}I-labeled DNA>

]

but use of <^{125}I-labeling complex /2,2/> and <^{125}I-labeling complex /1,3/> are also disclosed.

The overall process of analysis using such a <^{125}I-labeling complex> based reusable diagnostic P-chip is as follows:

*a. **Protein isolation;***

*b. **Protein purification;***

*c. **Protein capture on array of Ab1(i);***

*d. **Stringent wash and blocking;***

*e. **Conjugation of library of biotinylated Ab2(i);***

*f. **Stringent wash and blocking;***

*g. **The labeling via <125I- labeling complex>;***

*h. **Stringent wash;***

*i. **Counting and analysis:*** The MPD multi-well counters have very high sensitivity for ^{125}I decays and so the counter will introduce essentially no error to quantitation.

*j. **Cleavage using a restriction enzyme.***

*k. **Stringent wash and storage:*** Note, that in this implementation, no radioactive label is left after the cleavage step. Thus, the storage may be easier than in the first two methods of implementing reusable P-chips.

Note, that this implements reusable diagnostic P-chips which are not radioactive, and then perform the step of labeling *via* a universal radiolabeled streptavidin based complex. Thus, the use of radioactivity is limited to a short time during counting.

Optionally, the <labeling complex> may be labeled with a fluorescent or enzymatic label, but then the sensitivity of detection will be much lower.

II.7.3 Reusable P-chips with the step of fluor quenching.

We disclose an implementation of reusable diagnostic P-chip which is compatible with fluorescent labeling. Herein, we use the labeling complex:

]

< fl-labeling complex /3,1/> =]<streptavidin>[<fl-terminal>

]

but use of <fl-labeling complex /2,2/> and <fl-labeling complex /1,3/> is also disclosed.

Herein, the <fl-terminal> is an arbitrary length dsDNA which is biotinylated at 3" and is labeled with quenchable fluorescent label. We will also use a universal <fluor label quencher> also called <fl-quencher>, *i.e.* oligonucleotide complementary to <fl-terminal> and able to quench the fluorescent label on <fl-terminal>.

The overall process of analysis for reusable P-chip based on activated/deactivated fluorescent labeling " is as follow:

a. Proteins isolation;

b. Proteins purification;

c. Proteins capture on array of Ab1(i);

d. Stringent wash and blocking;

e. Conjugation of library of biotinylated Ab2(i);

f. Stringent wash and blocking;

j. The labeling via <fl-labeling complex>:

f. Stringent wash;

g. Counting and analysis: A multi-well fluorimeter will be used.

h. The hybridization of <fl-quencher>:

i. Stringent wash and storage: Note, that in this implementation, no radioactive label is used, which facilitates storage.

II.7.4 Reusable P-chip using resonant energy transfer.

This implementation is somewhat similar to II.7.3, but instead of the fluorescent label, a resonant energy transfer mechanism is used. The two DNA strands in the <RET terminal> are separated with peptide nucleic acid (PNA) openers to quench the signal,.

We disclose an implementation of reusable P-chip that is compatible with resonant energy transfer (RET) labeling. The following labeling complex is used:

]

< RET-labeling complex /3,1/> =]<streptavidin>[<RET-terminal>

]

The <RET-terminal> is an arbitrary length dsDNA that is biotinylated at its 3? end and is labeled with a RET label. We will also use an PNA opener which leads to creation of PNA bubble which blocks the resonance energy transfer.

Obviously, the similar <RET-labeling complex/2,2/> and <RET-labeling complex/1,3/> can be used as well.

The overall process of analysis for reusable P-chip based on activated/deactivated RET labeling is as follow:

a. Protein isolation;

b. Protein purification;

c. Protein capture on array of Ab1(i);

d. Stringent wash and blocking;

e. Conjugation of library of biotinylated Ab2(i);

f. The labeling via <RET labeling complex>: This is a classical techniques used in many immunologic assays, *e.g.* ELISA. However, the optimization of conditions (incubation time, incubation temperature, pH) is crucial to diminish NSBB.

g. Counting and analysis:

h. The hybridization of PNA openers and creation of a PNA bubble.

i. Stringent wash and storage: Note, that in this implementation, no radioactive label is used. Thus, the storage may be easier than in two first methods implementing reusable P-chips.

II.8 Reusable P-chip using a thermally decoupled linker.

In many cases, the simple thermal cycling of a particular labeled streptavidin-ds-DNA construct, would be a less costly alternative than the five implementations disclosed above. Such a procedure will be facilitated by existence of many thermal cyclers, mostly used in PCR.

In this implementation, the <streptavidin- dsDNA> construct is used, wherein the special structure of dsDNA is used, namely

```
                < ssDNA1 >        < dsDNA2 >            < dsDNA3 >
<dsDNA> = *************          **************        **************
          <compl-ssDNA1><polyA><compl-ssDNA2><polyAA><compl-dsDNA3>
```

and the three parts <ssDNA1>, <ssDNA2> and <dsDNA3> have a particular form. In the following these three parts are called <biotinylated linker>, spacer> and <labeling probe>, respectively.

Thus the structure of <dsDNA1> is:

<biotinylated ssDNA>

<dsDNA1> = ******************

< ssDNA >

i.e. the biotinylated on 5' ssDNA is hybridized with complementary polynucleotides. The <dsDNA2> has a particular structure of

< non -iodinated ssDNA>

$<dsDNA2>_{AT}$ = **********************

< non-iodinated ssDNA >

wherein $<dsDNA2>_{AT}$ is A and T rich.

Also, <dsDNA3> has a particular structure

< iodinated ssDNA >

$<dsDNA3>_{CG}$= ***********************

< non-iodinated ssDNA >

wherein $<dsDNA3>_{CG}$ is C and G rich.

Herein, the melting temperature of the first fragment is selected to be quite high, *i.e.* the length is preferably longer than 20-mer, and the sequence of this fragment contains majority of CG

bonds, which are characterized by high melting temperature. In contrast, <dsDNA2> and <dsDNA3> are selected as low melting temperature complexes.

Applications the thermal cycling (like in reusable DNA chips) using this construct, *i.e.* use of a temperature high enough to destroy stability of ds-DNA is possible. However, the use of such procedures is not possible when implementing reusable P-chip. To achieve the good stability of hybridization, appropriate 20-30 mers are used. However, the melting temperature of such double stranded fragment is typically above 60 oC. We note, that the melting temperature (T_m) is herein defined as a temperature in which 50% of the ds-polynucleotides are decoupled into two complementary strands. To achieve the high melting fraction, say 90-95% as required in applications to reusable protein chips, one would need to use a temperature above 80% or cycle to lower temperature, say 60oC many times. However, such a cycling will lead to denaturation of the Abs and induce very large drifts in the reusable P-chip performance. We note that to be practicable, the CV of reusable P-chip should be better than 20% after at least 20 cyclings. Thus, any drift of the avidity of reusable P-chip larger than 1% per cycle is not acceptable.

Thus, the three parts of DNA construct each have a particular function: <biotinylated linker>, <spacer>, <labeling probe>, but also particular, highly different thermal cycling properties.

Herein, the melting temperature of the first fragments <dsDNA1> is selected to be quite high, *i.e.* the length is preferably longer than 20-mer, and the sequence of this fragment contains majority of CG pairings, which are characterized by high melting temperature. In contrast, <dsDNA2> and <dsDNA3> are selected with a low melting temperature. A possible implementation would be to select short ssdNA fragments, say 10-15 mers and use only AT pairings which has relatively low melting temperature. However, in this case radioiodination is difficult, because we use ^{125}I-dCTP as labeling moiety.

Thus, in a preferred implementation, one needs to use a very particular structure of the <dsDNA2> wherein, the lower strand is G-rich, but a modified G-nucleotide is used. By attaching of particular covalent adducts to G, *e.g.* by fluorinating G, the melting temperature of C-$G_{modified}$ can be considerably diminished.

An example of such structure is

<dsDNA>=bio-CGCGCGCGCGCGCGCGCGCGC TTTTTTTTTTTT *CTCACTCACTCA*

******************** ************* *************

GCGCGCGCGCGCGCGCGCGCGAAAAAAAAAAAAAAAAGAGTGAGTGAGT

wherein we selected the simplest implementation of A and T rich structure, *i.e.* one of the strands is a pure polyA. In the above the bases shown in italics are modified, namely "*C*" are radioiodinated.

There is however, a second reason why even the quite artificial structure above may not be adequate. In the reusable P-chip concept, the streptavidin stays attached to the secondary Ab *via* biotin/streptavidin coupling. Thus, after the thermal cycling the stripped <dsDNA> is still present and has the structure:

<dsDNA>$_s$ = bio-CGCGCGCGCGCGCGCGCGCGC

****

CGCGCGCGCGCGCGCGCGAAGAGTGAGTGAGTGAAGAGTGAGTGAGTGA

stays attached to the streptavidin. Thus, in next step of probing, the radioiodinated ssDNA fragment becomes hybridized, removing the advantage of reusability. Alas, after the step of thermal cycling and washing the radiolabeled probe, the step of blocking has to be performed. One can not use the same structure as when blocking, because they will have the same melting temperature and will compete in the next probing.

This is a justification of using three part structure of <dsDNA>, wherein we intentionally induced gaps between the two hybridizing polynucleotides. Alas, one can not use the same structure as in the probing. However, by using a simple reagent which consists of

<ssDNA2><polyT><ssDNA3>, the very high melting temperature is obtained, because the final product is both long as well as C and G rich. Thus, in the example above the blocking probe is

<blocking probe> = TT<ssDNA2>TT<ssDNA3>=

TTTTTTTTTTTTTTTCTCACTCACTCACT

Note, that in first part of <blocking part> has additional four T, so that there is no nick after hybridization. Also, the noniodinated C are used interspersed with T. Observe that after hybridization of blocking probe, both nicks are removed, *i.e.* a reasonably long (in our example 30-mer) and CG rich structure is obtained. Thus, the estimated melting temperature of blocking probe is about 30oC higher than either of <spacer> or <labeling probe>.

Another implementation of the system is disclosed. In this case, the blocking probe consists of PNA complementary to the lower strand of <spacer><labeling probe>. The PNA/DNA hybrids have much higher melting temperature than DNA/DNA complexes.

More generally, we describe the special construct, in which the DSDNA attached to streptavidin has a very particular structure, in which biotinylated part has very high melting temperature and the <spacer><labeling probe> has a very low melting temperature.

The overall process of analysis for reusable P-chip based on active/deactivated RET labeling is as follow:

a. Protein isolation;

b. Protein purification;

c. Protein capture on array of Ab1(i);

d. Stringent wash and blocking;

e. Conjugation of library of biotinylated Ab2(i);

j. The labeling via <labeling probe complex>:

k. Counting and analysis:

l. The thermal cycling leading to melting of <spacer><labeling probe>;

m. Stringent wash:

n. Blocking with <blocking probe>

o. Storage

CLAIMS:

I claim:

1. A reusable protein chip which can quantitate at least a few hundreds of proteins with a sensitivity not worse than 10 pg/ml.

2. A protein chip according to claim 1, wherein the said target proteins are captured on spatially resolve pads.

3. A protein chip according to claim 2, wherein the said pads are of identical dimensions and ordered in array pattern.

4. A protein chip according to claim 2, wherein the majority of said pads are of identical dimensions but some are much larger to permit binding of more abundant proteins.

5. A protein chip according to claims 1 and 2, wherein the said pads are covered with a target specific moities at at least 1,000 excess to the expected level of the targeted proteins.

6. A protein chip according to claim 5, wherein the said target specific moieties are antibodies.

7. A protein chip according to claim 5, wherein the said target specific moieties are antibodies produced by phage display.

8. A protein chip according to claim 5, wherein the said target specific moieties are aptamers.

9. A protein chip according to claim 5, wherein the said target specific moieties are antibodies or phage display antibodies or aptamers, and wherein type of the capturing moietie is selected to provide the highest possible specificity.

10. A reusable protein chip according to claim 1, wherein the said proteins are radiolabeled.

11. A reusable protein chip according to claim 1, wherein the said proteins are radioiodinated.

12. A reusable protein chip according to claim 1, wherein the said proteins are ^{123}I-labeled.

13. A protein binding, reusable protein chip according to claim 1, wherein after the said proteins are ^{125}I-labeled.

14. A reusable protein chip according to claim 1, wherein the first step of the procedure is fractionation by abundance (FbA), *i.e.* the most abundant proteins are rejected before step of capture.

15. A method of using a reusable protein chip comprising:

a. Protein extraction:
b. Removal of the most abundant proteins:
c. Protein denaturation (this step is optional);
d. Proteins labeling with ^{123}I;
e. Protein capture;
f. Stringent wash;
g. Counting and analysis;
h. At least 48 hours storage before next use.

16. A method of using a reusable P-chip using ^{123}I according to claim 1, which involves the step of proteins biotinylation and subsequent use of ^{123}I-streptavidin.

a. Protein isolation;

b. Removal of the most abundant proteins;

c. Protein denaturation (this step is optional);

d. Protein biotinylation;

e. Protein capture:

f. Stringent wash and blocking followed by stringent wash;

g. The labeling *via* biotin-^{123}I streptavidin coupling;

h. Counting and analysis;

i. At least 48 hours storage before next use.

17. A method according to claim 16, wherein MPD detectors are used to quantitate ^{123}I.

18. A method according to claim 16, wherein the storage of the chip is performed in special dialysis chamber in presence of free radicals scavengers.

19. A method of using a reusable P-chip with radiolabeling and use of cleavage by restriction enzyme(s).

20. A new labeling probe with the generic structure of:

<labeling complex > =]<streptavidin>[<DNA complex>

wherein

<DNA complex> = < biotinylated DNA><switch><labeled DNA>

21. A probe according to claim 20, wherein:

<DNA complex> = <biotinylated AT linker><AT switch>< radiolabeled terminal>, both the <biotinylated AT linker> and the <AT switch> contain no C or G bases, whereas <radiolabeled terminal> is CG rich.

22. A method of using a reusable protein chip according to claim 19, using "enzymatic cleavage" as follows:

a. Protein isolation;
b. Removal of the most abundant proteins;
c. Proteins biotinylation;
d. Proteins capture;
e. Stringent wash and blocking;
f. The labeling via <labeling complex>;
h. Counting and analysis;
i. Cleavage using a restriction enzyme;
j. Stringent wash and storage.

23. An implementation of reusable P-chip with use of fluorescent label and special fluorescence quencher.

24. A new labeling probe with the generic structure of:

<fl-labeling complex > =]<streptavidin>[<fl-DNA complex>

wherein

<DNA complex> = <biotinylated DNA><fluorescent ssDNA>

and wherein a special <fluorescence quencher> is appropriately modified ssDNA oligonucleotide complementary to fluorescent ssDNA.

25. A method of using the reusable P-chip according to 24, wherein the following steps are used:

a. Protein isolation;
b. Removal of the most abundant proteins;
c. Proteins biotinylation;
d. Proteins capture;

e. Stringent wash and blocking;

f. The labeling via <fl-labeling complex>;

g. Counting and analysis;

j. The hybridization of <fl-quencher>;

k. Stringent wash and storage.

26. An implementation of reusable P-chip with use of resonace energy transfer (RET) and PNA openers.

27. A new labeling probe with the generic structure of:

<RET-labeling complex > =]<streptavidin>[<RET-DNA complex>

wherein

<RET-DNA complex> = <biotinylated DNA><RET labeled DNA>

and wherein PNA openers are used to deactivate <RET labeled DNA>.

28. A method of using the resusable P-chip according to 24, wherein the following steps are used:

a. Protein isolation;

b. Removal of the most abundant proteins;

c. Proteins biotinylation;

d. Proteins capture;

e. Stringent wash and blocking;

f. The labeling *via* <RET-labeling complex>;

g. Counting and analysis;

j. The deactivation of RET label by hybridization of PNA openers;

k. Stringent wash and storage.

29. A method of using a reusable P-chip in "Ab1-P-Ab2 sandwich format" with sensitivity better than 100 fg/ml, wherein the libraries of capture antibodies {Ab1(i)} and labeling antibodies {Ab2(i)} are used.

30. The method according to claim 29, wherein the said labeling antibodies {Ab2(i)} are radiolabeled.

31. The method according to claim 30, wherein the said labeling antibodies {Ab2(i)} are labeled with short life time radioisotope, with a prefered implementation wherein the said short life-time radioisotope is ^{123}I.

32. The method according to claim 31, wherein the following procedure is used:

a. Protein isolation;
b. Protein purification;
c. Proteins capture;
d. Stringent wash and blocking;
e. Conjugation of library of ^{123}I labeled antibodies Ab2(i);
f. Stringent wash;
l. Counting and analysis;
i. At least 48 hours storage before next use.

33. A method according to claim 32 wherein the step of quantification uses the MPD instrumentation

34. A method according to claim 29, with radiolabeling via <labeling complex> and subsequent use of cleavage by restriction enzyme(s).

35. A particular implementation of resusable P-chip according to claim 34, wherein the labeling complex according to claim 20 is used.

36. The method of claim 19, wherein the process of analysis using such "enzymatic cleavage" based reusable P-chip is as follows:

a. Protein isolation;

b. Removal of the most abundant proteins;

c. Proteins capture;

d. Stringent wash and blocking;

e. Conjugation of library of biotinylated antibodies Ab2(i);

f. Stringent wash and blocking;

g. The labeling *via* <labeling complex>;

h. Stringent wash;

i. Counting and analysis;

j. Cleavage using a restriction enzyme;

k. Stringent wash and storage.

l. Storage

37. The method according to claim 29 with use of special labeling complex including fluorescent label and a subsequent use of fluorescence quencher.

38. The method according to claim 37, wherein the <fl-labeling complex> according to claim 24 is used.

39. The method of claim 37, wherein the following steps are used:

a. Protein isolation;

b. Removal of the most abundant proteins;

c. Proteins capture;

e. Stringent wash and blocking;

f. Conjugation of library of biotinylated {Ab2(i)};

g. Stringent wash and blocking;

h. The labeling *via* <fl-labeling complex>;

i. Counting and analysis;

j. The hybridization of <fl-quencher>;

k. Stringent wash and storage.

40. The method of claim 29 with use of special labeling complex including resonant energy transfer label and a subsequent use of PNA openers.

41. The method of claim 40, wherein the <RET-labeling complex> according to claim 27 is used.

42. The method of claim 41, comprising the following steps:

a. Protein isolation;
b. Removal of the most abundant proteins;
c. Proteins capture;
d. Stringent wash and blocking;
e. Conjugation of biotinylated {Ab2(i)};
f. Stringent wash and blocking;
g. The labeling *via* <RET-labeling complex>;
h. Counting and analysis;
i. The deactivation of RET label by hybridization of PNA openers;
j. Stringent wash and storage.

43. A method of using a reusable P-chip in "Ap-P-Ab sandwich format" with sensitivity better than 100 fg/ml, wherein the libraries of aptamers {Ap(i)} and antibodies {Ab(i)} are used as capturing and labeling moieties, respectively.

44. The method of claim 43, wherein the said library of antibodies is labeled with short life isotopes, *e.g.* ^{123}I.

45. The method of claim 43, wherein the said antibodies are biotinylated and the <radiolabeled labeling complex> including a <switch> and subsequently restriction enzyme are used.

46. The method of claim 43, wherein the said antibodies are biotinylated and the <fl-labeling complex> and subsequently <fl-quencher> are used.

47. The method of claim 43, wherein the said antibodies are biotinylated and the <RET-labeling complex> and subsequently PNA openers are used.

48. A method of using a reusable P-chip in "Ab-P-Ap sandwich format" with sensitivity better than 100 fg/ml, wherein the libraries of antibodies {Ab(i)} and aptamers {Ab(i)} are used as capturing and labeling moieties, respectively.

49. The method of claim 48, wherein the said aptamers are labeled with short life isotopes, *e.g.* ^{123}I.

50. The method of claim 48, wherein the said aptamers are biotinylated and the <radiolabeled labeling complex> including a <switch> and subsequently restriction enzyme are used.

51. The method of claim 48, wherein the said aptamers are biotinylated and the <fl-labeling complex> and subsequently <fl-quencher> are used.

52. The method of claim 48, wherein the said aptamers are biotinylated and the <RET-labeling complex> and subsequently PNA openers are used.

53. A method of using a reusable P-chip wherein dsDNA attached to streptavidin permits eliminating the label by controlled heating and subsequent wash.

54. A method of using a P-chip for low abundance protein, wherein the sensitivity of the P-chip is better than 100 fg/ml for a majority of targets.

55. The method of claim 54, wherein the sensitivity is achieved by the use of MPD instrumentation.

56. The method of claim 54, wherein the sensitivity is achieved by division of P-chips into three zones, dedicated to quantitation of high abundance proteins (HAPs), medium abundance proteins (MAPs) and low abundance proteins (LAPs), respectively.

57. The method of claim 54 wherein the sensitivity is achieved by division of P-chips into three zones, each zone featuring predominantly the target specific moieties of different type, *e.g.* phage display Abs for HAps, Aptomers for MAPs and hybridoma generated Abs for LAPs.

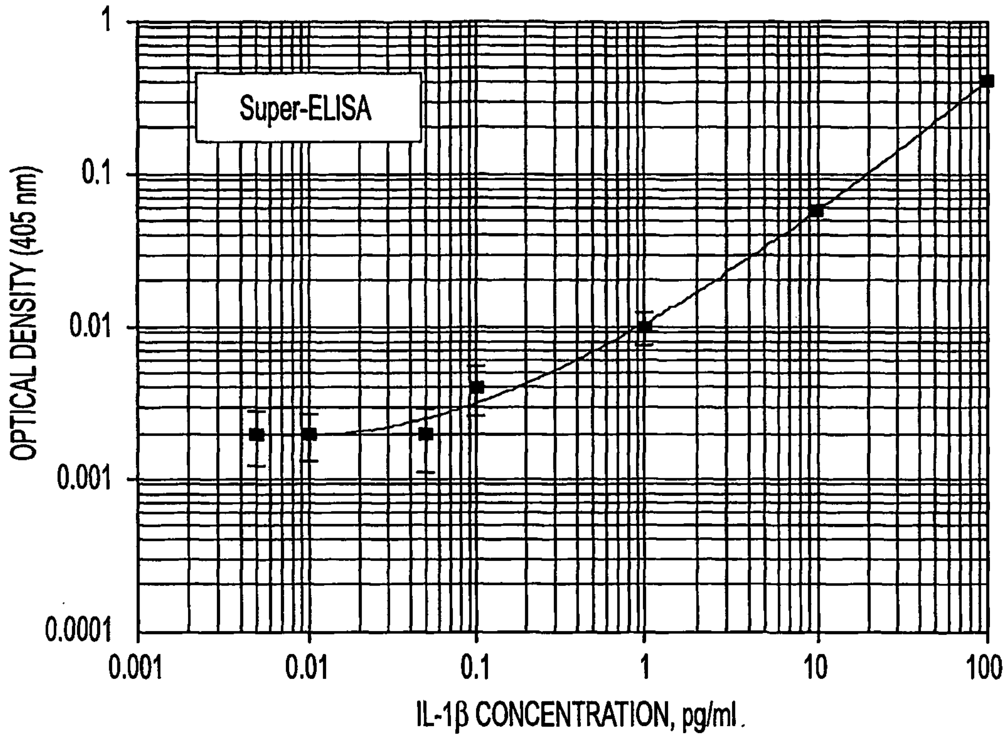
58. The method of claim 54, wherein the sensitivity is achieved by division of P-chips into three zones, each zone featuring predominantly the pixels of different size, largest for HAPs, smaller for MAps and sub-millimeter in diameter for LAPs.

59. The method of claim 54, wherein the sensitivity is achieved by division of P-chips into three zones, each zone featuring predominantly the pixels of different size: largest for HAPs, smaller for MAps and sub-millimeter in diameter for LAPs.

60. The method of claim 54, wherein the sensitivity is achieved by division of P-chips into three zones for HAPs, MAPs and LAPS, respectively and for purpose of non-specific biological background (NSBB) removal the biological sample is first disposed in the first zone and after removal of HAPS, moved to second zone and after removal of MAPs to the third zone featuring predominantly the pixels of different size: largest for HAPs, smaller for MAps and sub-millimeter in diameter for LAPs.

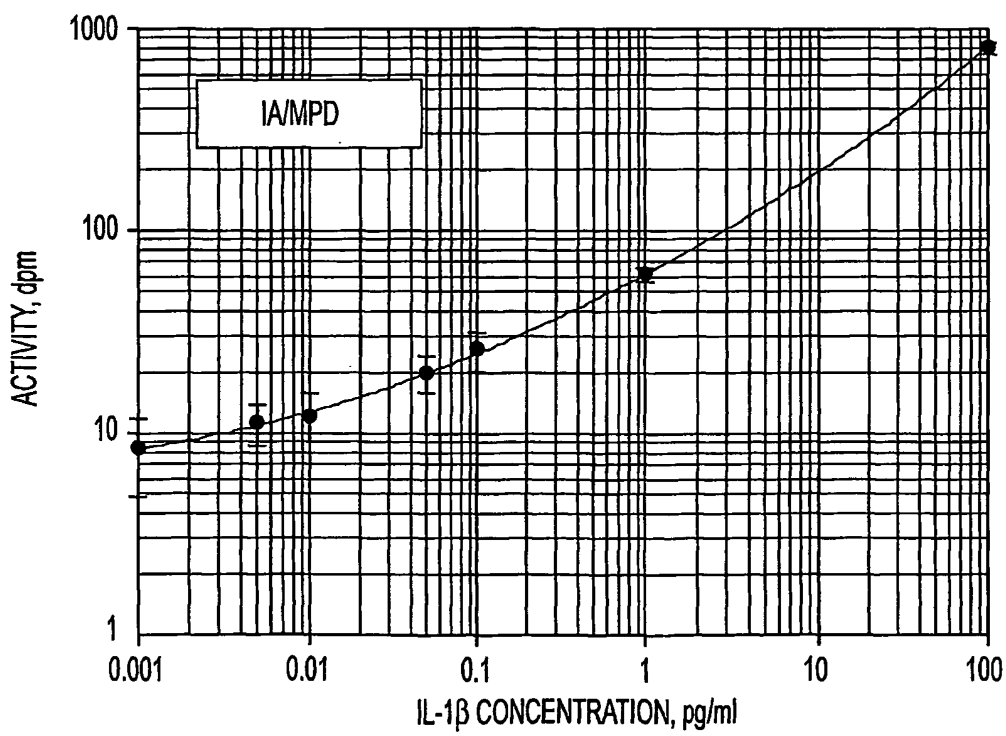
61. The method of claim 54, wherein the sensitivity is achieved by division of P-chips into three zones for HAPs, MAPs and LAPS, respectively and for purpose of non-specific biological losses removal (NSBL) removal the biological sample is first doposed in the LAPs zone and after moved to Maps zone and finally displaced to HAPs zone.

62. A procedure for measuring protein abundance in a biological sample, wherein the said sample is aliqoted into two, and parallel processed according to claims 60 or 61, and the results are compared to obtain an unbiased estimator taking into account both NSBB and NSBL.



Super-ELISA
OPTICAL DENSITY (405 nm)
1
0.1
0.01
0.001
0.0001
0.001
0.01
0.1
1
10
100
IL-1β CONCENTRATION, pg/ml

FIG. 1



IA/MPD
ACTIVITY, dpm
1000
100
10
1
0.001
0.01
0.1
1
10
100
IL-1β CONCENTRATION, pg/ml

FIG. 2

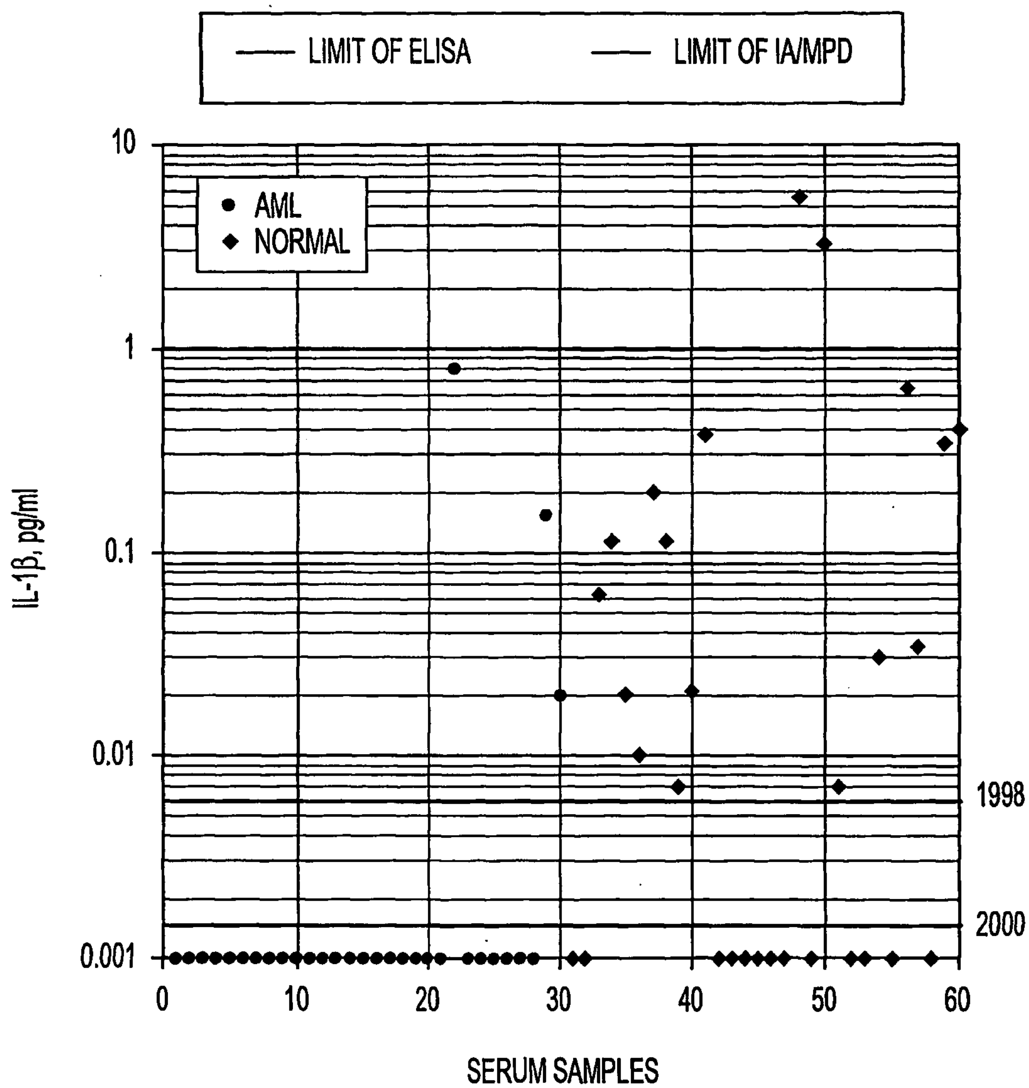

LIMIT OF ELISA
LIMIT OF IA/MPD
AML
NORMAL
10
1
0.1
0.01
0.001
1998
2000
IL-1β, pg/ml
0
10
20
30
40
50
60
SERUM SAMPLES

FIG. 3

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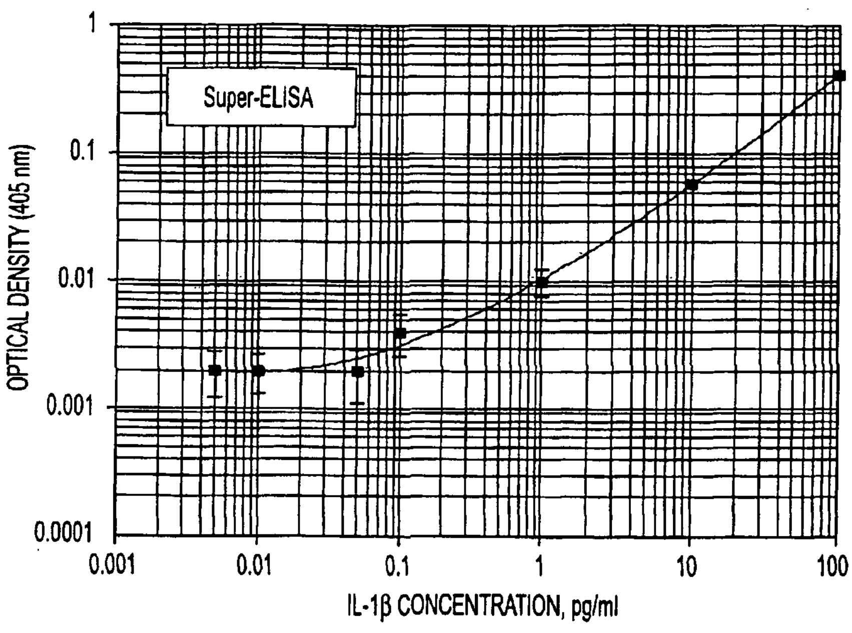
[Continued on next page]

(54) Title: REUSABLE MICROARRAYS FOR QUANTIFYING LOW ABUNDANCE PROTEINS



WO 01/081924 A3

(57) Abstract: The invention provides protein chip microarrays (P-chips) capable of detecting low abundance proteins from physiologic fluids that exist in concentrations smaller than 0.1 pg/ml. Quantitation is carried out by detecting multiphoton emitting radioisotopes from the assay. The method of multi photon detection provides P-chips with sensitivity of about 50 fg/ml, i.e. about 1,000 fold better than prior art P-chips. Cost effective reusable P-chips and methods of using them are also provided.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 01/13025

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C12N15/10 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 43287 A (DRUKIER ANDRZEJ K ;BIOTRACES INC (US); WILK ANDRZEJ (US)) 2 September 1999 (1999-09-02)	15-25, 36,53,62
Y		1-14, 26-35, 37-52, 54-61
	page 6, line 10 -page 9, line 29 claims ---	
Y	EP 1 006 362 A (CAHILL MICHAEL DR ;NORDHEIM ALFRED PROF DR (DE); DRUKIER ANDRZEJ D) 7 June 2000 (2000-06-07) paragraphs ‘0012!-‘0014! paragraphs ‘0031!,‘0035!,‘0054! claims 20-23 -----	1-14, 26-35, 37-52, 54-61

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 November 2002	29/11/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stricker, J-E

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/13025

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9943287	A	02-09-1999	AU	2788099 A	15-09-1999
			WO	9943287 A2	02-09-1999
EP 1006362	A	07-06-2000	EP	1006362 A1	07-06-2000
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